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Development of a multicellular co-culture model of normal and cystic fibrosis human airways in vitro

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DEVELOPMENT OF A MULTICELLULAR CO-CULTURE MODEL OF NORMAL AND CYSTIC FIBROSIS HUMAN AIRWAYS *IN VITRO*

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Summary

Cystic fibrosis (CF) is the most common lethal inherited disease among Caucasians and arises due to mutations in a chloride channel, called cystic fibrosis transmembrane conductance regulator. A hallmark of this disease is the chronic bacterial infection of the airways, which is usually, associated with pathogens such as *Pseudomonas aeruginosa, S. aureus* and recently becoming more prominent, *B. cepacia*. The excessive inflammatory response, which leads to irreversible lung damage, will in the long term lead to mortality of the patient at around the age of 40 years.
Understanding the pathogenesis of CF currently relies on animal models, such as those employing genetically-modified mice, and on single cell culture models, which are grown either as polarised or non-polarised epithelium *in vitro*. Whilst these approaches partially enable the study of disease progression in CF, both types of models have inherent limitations.
The overall aim of this thesis was to establish a multicellular co-culture model of normal and CF human airways *in vitro*, which helps to partially overcome these limitations and permits analysis of cell-to-cell communication in the airways. These models could then be used to examine the co-ordinated response of the airways to infection with relevant pathogens in order to validate this approach over animals/single cell models. Therefore epithelial cell lines of non-CF and CF background were employed in a co-culture model together with human pulmonary fibroblasts. Co-cultures were grown on collagen-coated permeable supports at air-liquid interface to promote epithelial cell differentiation. The models were characterised and essential features for investigating CF infections and inflammatory responses were investigated and analysed. A pseudostratified like epithelial cell layer was established at air liquid interface (ALI) of mono-and co-cultures and cell layer integrity was verified by tight junction (TJ) staining and transepithelial resistance measurements (TER). Mono- and co-cultures were also found to secrete the airway mucin MUC5AC. Influence of bacterial infections was found to be most challenging when intact *S. aureus*, *B. cepacia* and *P. aeruginosa* were used. CF mono- and co-cultures were found to mimic the hyperinflammatory state found in CF, which was confirmed by analysing IL-8 secretions of these models.
These co-culture models will help to elucidate the role fibroblasts play in the inflammatory response to bacteria and will provide a useful testing platform to further investigate the dysregulated airway responses seen in CF.

Key words: Cystic Fibrosis, *in vitro*, co-culture, air-liquid interface, human pulmonary fibroblasts

For my brother Tim

And

My lovely parents

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Abbreviations

AAVCFTR	adeno-associated viral CFTR
AEM	Airway epithelial cell growth medium
ALI	Air-liquid interface
ASL	Air surface liquid
BMZ	Basement membrane zone
CF	Cystic Firbrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CHO	Chinese hamster ovary cells
CK	Cytokeratin
Cl^-	Chloride ion
cm	Centimetre
COPD	Chronic obstructive pulmonary disease
CTB	Cell titer blue
DAPI	4’,6 diamidino-2-phenylindole
DMEM/F12	Dulbecco’s modified eagle medium: Nutrient mixture F-12
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylendiaminetetraacetic acid

FBS	Fetal bovine serum
FITC	Fluorescein isothio-cyanate
GAG	Glycosaminoglycans
H & E	Hematoxylin and eosin
HIA	Heat inactivated
HPF	Human pulmonary fibroblast
HPFM	Human pulmonary fibroblast growth medium
Ig	Immunoglobulin
IL-8	Interleukin 8
IMS	Industrial methylated spirit
ITS	Insulin-Transferrin-Selenium Growth supplement
KC	Keratinocyte chemokine
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MCC	Mucociliary clearance
mg	Milligram
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIP-2	Macrophage inflammatory protein 2
ml	Millilitre
mm	Millimetre
MMP	Metalloproteinase

MV	Microvilli
Na^+	Sodium ion
Ng	Nanogram
OD	Optical density
PAMP	Pathogen-associated pattern
PBS	Phosphate buffered saline
PCL	Periciliary liquid
PE	Phycoerithrin
PET	Polyethylene terephthalate
pg	picogram
PGN	Proteoglycan
PI	Propidium Iodide
PRR	Pattern recognition receptor
SEM	Scanning electron microscopy
SF	Serum free
SG	Secretory granules
TEM	Transmission electron microscopy
TER	Transepithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor
TW(s)	Transwell insert (s)
ZO-1	Zonula occludens

1 Chapter 1 Introduction

1.1 The human respiratory system

The human respiratory system consists of two regions, the upper and the lower airways.

The upper airways consist of nose, oral cavity, pharynx and larynx (figure 1.1) and the main functions of the upper airways are to humidify, warm and filter inhaled air to mostly prevent foreign particles, including viruses and bacteria, from entering the tracheobronchial tree.

The lower airways or tracheobronchial tree consist of a series of branching airways, called generations or orders. The right lung has three lobes, whereas the left lung only has two lobes. The trachea bifurcates into right and left main bronchi, which branch into secondary (lobar) bronchi, then divide again into tertiary (segmental) bronchi before finally branching into bronchioles, which are the smallest airways without alveoli. This part of the lower airways is called the conducting airway region, which leads inspired air to the gas exchanging regions downstream, the alveolar ducts. The alveolar ducts are lined with alveolar sacs (alveoli).The entire respiratory system is lined with epithelial cells containing several different cell types, which all have specific structure and function.

Figure 1.1 The anatomy of human airways (Anon, 2011)

1.1.1 The structure of the airway epithelium

The respiratory tract is composed of several different epithelial cell types and each fulfils certain functions to maintain lung homeostasis (Breeze and Wheeldon, 1977). Different epithelial cell types have been described and can be classified into the following three major categories: basal, ciliated columnar and non-ciliated secretory columnar cells. Other cells that can be found in the respiratory system are immune and inflammatory cells, which can migrate to the airways through the basement membrane to support epithelial cell function (Knight and Holgate, 2003). The cross section of the airway wall shows the different epithelial cell types and their position on top of the underlying basement membrane. The bronchial epithelium is organised as a pseudostratified cell layer comprised of basal, ciliated and secretory cells (goblet). The basal cell population helps to build up the pseudostratified appearance as they are strongly attached to the basement membrane through hemidesmosomes, which are specialized cell-extra cellular

matrix junctions (Green and Jones 1996). Additionally desmosomes, which are intercellular adhesion molecules create trans-cellular networks and give the tissue a high resistance to mechanical stress (Knight and Holgate, 2003). Furthermore there are tight junctions, who ensure columnar-columnar interactions and also they form a tight but selective barrier in the paracellular space between epithelial cells, separating the lumen of the airways from the underlying tissue. Tight junctions are located closest to the lumen and form a belt like appearance where adjoining cells are connected to each other's membrane (Farquhar and Palade, 1963).

1.1.1.1 Cells of the airway epithelium

Basal cells are abundant in the conducting airways although their number decreases according to the size of the region. There are much more numerous in the trachea and extrapulmonary bronchi (Jeffery and Reid, 1975). Basal cells are important for attaching other epithelial cells to the basement membrane (BM) *via* hemidesmosomes. They have pyramidal or ovoid shape containing a large nucleus, which is surrounded by low amount of cytoplasm. Historically, basal cells were thought to be the origin of stem cells in the airway epithelium, giving rise to ciliated and secretory columnar cells in larger airways (Breeze and Wheeldon, 1977). In addition to their possible progenitor role and attachment of superficial cells to the basement membrane, basal cells also secrete a number of active molecules including cytokines, chemokines, and growth factors. In the pseudostratified epithelium all cells rest on the basement membrane but basal cells do not reach the lumen and do not contribute to the apical epithelial surface.

Secretory cells, such as goblet cells and serous cell, contribute to the secretion of airway mucus and they are present in the apical surface of the epithelial cell layer. Goblet cells are most numerous and the main source of airway mucus. They are characterised by the electron-lucent appearance of secretory granules and morphologically show microvilli expression on the cell surface. Additionally they take part in the inflammatory response by rapidly increasing mucus secretion after exposure to bacterial infection, for example (Gail and Lenfant, 1983, Jeffery and Li, 1997). A thin mucus layer lines the epithelium as an innate defence mechanism, which entraps any inhaled particles, other foreign molecules, bacteria and viruses. This is part of the mucociliary escalator, which will be discussed further on. Serous cells are another type of secretory cell, resembling mucus goblet cells with a difference in granule content where it is seen to be electron-dense. These cells have only been found in rodent airways, but two populations of these rare cells have been observed in small airways of human lung (Rogers *et al*., 1993).

Columnar ciliated cells, found in the pseudostratified epithelium, reach the lumen but still attach to the basement membrane and they form tight junctions with other columnar cells but also form desmosomes to attach to adjacent cells and basal cells (Breeze and Wheeldon, 1977, Farquhar and Palade, 1963). Furthermore they directly take part in the mucociliary escalator, which was just mentioned above as these cells, which make up 50 % of all epithelial cells in the airways (Knight and Holgate, 2003), are responsible for moving the mucus blanket towards the pharynx. All entrapped bacteria and particles are eliminated in this way in healthy airways.

1.1.2 Pulmonay Fibroblasts

Pulmonary fibroblasts were long thought to only be an inert structural supporting cell but growing evidence has been reported and confirmed that these cells can actively contribute directly to pulmonary inflammation and they are also responsible for deposition of the ECM within the lung (McAnulty *et al*., 1995, Mutsaers *et al*., 1997). Interstitial fibroblasts in the lungs account for about 40 % of all lung cells (Dunsmore and Rannels, 1996). In the conducting airways there are subepithelial fibroblasts, which are in close proximity to the epithelium, which allows direct close interaction between these two cell types and the ECM (Brewster *et al*., 1990). These mesenchymal cells were termed attenuated fibroblasts and together with the epithelium, the ECM and neural tissue this unit has been termed mesenchymal trophic unit (Evans *et al*., 1999). This complex has been shown to be important during airway growth and airway branching (Minoo and King, 1994). For asthma it has been shown that reactivation of this unit leads to fibrosis (Holgate *et al*., 2000). In CF it is thought that anti-fibrotic factors (Interferon-γ), which have been found to be increased in CF, lead to normal repair without fibrosis. CF tissue has been found to show normal histological appearance, even in the presence of infection (Shute *et al*., 2003).

1.1.3 The extracellular matrix (ECM) and basement membrane

The extracellular matrix (ECM) includes the interstitial matrix, which is present between the different cells in the intercellular spaces, and BM, which is a sheet-like deposition of ECM. The complex composition of the ECM is crucial in terms of displaying a highly organised part of connective tissue. It serves several different functions, such as support and anchorage for cells, segregating different tissues from one another and regulating intercellular communication. In the airways the ECM has to maintain normal lung functions by maintaining functional and structural integrity.

A constant balancing act is kept between synthesis and degradation of the different components of ECM. Three controlling mechanisms are responsible for this balance. One is de novo sythesis and deposition of ECM components, the second is proteolytic degradation of existing ECM by Matrix-Metalloproteinases (MMPs) and the last one is the inhibition of MMP activity by specific endogenous antiproteases, the tissue inhibitors of MMPs (TIMPs)(Eickelberg *et al*., 1999). The ECM consists mainly of fibrous proteins and glycosaminoglycans (GAGs) including proteoglycans, collagens, elastins, laminin and fibronectin, which are mostly produced by fibroblasts (Diaz *et al*., 1989, Eickelberg *et al*., 1999, Miyazaki *et al*., 1990).

The basement membrane (BM) also called basal lamina, is a thin sheet-like membrane (40-120nm), on which epithelial cells adhere. The BM mainly consists of type IV collagen, laminin, fibronectin, proteoglycans, including perlecan and provides structural support and it is presented in every tissue of the human body, where epithelial cells are always linked to it. The BM separates these epithelial cells from the underlying connective tissue but it also plays a role in epithelial-mesenchyme interactions and it has been found to be tissue specific. In addition the BM facilitates epithelial cell adhesion and migration through ECM interactions, plus it can also serve as a growth factor reservoir. Furthermore it plays a role in establishing epithelial cell polarity (LeBleu *et al*., 2007, Howat *et al*., 2001).

1.1.4 Function of the airway epithelium

The main function of the complex airway epithelium is to protect the airways and the underlying tissue from inhaled airborne particles, noxious substances and pathogens. As earlier mentioned the airway epithelium is the first line of contact with the outside environment and it is therefore responsible to entrap the inhaled foreign particles in the luminal mucus layer, which covers the bronchial epithelium, and that way the mixture will be removed from the lung by coordinated directional ciliary beating (mucociliary escalator) moving the mucus and trapped particles towards the pharynx. This defence mechanism is responsible for keeping the airway mostly sterile. If this mechanism fails and a particle or pathogen is recognised by the host immune system, a pro-inflammatory signalling pathway is activated, upon which the epithelial cells produce and release inflammatory cytokines, such as IL-8 (Kim *et al*., 1997, Abdullah and Davis, 2007, Livraghi and Randell, 2007, Mills *et al*., 1999, Atsuta *et al*., 1997)

1.1.4.1 Mucociliary clearance (MCC, or mucociliary escalator)

MCC of the respiratory tract is an important innate defence mechanism against inhaled particles and pathogens. Cilia lining the upper and lower airways are covered by a thin mucus layer and beat rapidly in a coordinated direction propelling mucus and trapped particles towards the pharynx (Gail and Lenfant, 1983). The air-surface liquid (ASL) is crucial for effective mucus clearance, which consists of a periciliary liquid (PCL) layer and a mucus layer overlying the PCL. The PCL keeps the mucus the right distance away from the underlying epithelia, this is usually about 7µm, the same length as the cilia, whereas the mucus layer can vary in height from 7 µm - 70 µm (Tarran, 2004). The PCL provides a low viscosity solution that lubricates the cilia, which then can beat rapidly and continuously. The mucus layer, as described above, is responsible for entrapping any inhaled particles and substances to protect the lung from infection and inflammation. The viscoelastic mucus layer consists of a mixture of water, ions and mainly of two of the oligomeric glycoprotein mucins, MUC5AC and MUC5B (Thornton and Sheehan, 2004, Breeze and Wheeldon, 1977, Boucher, 2007). Moreover, the airway epithelium serves as a rich source of anti-oxidants and anti-bacterial agents including lactoferrin, lysozyme, and opsonins (Velden and Versnel 1998). It also produces anti-proteases such as tissue inhibitors of metalloproteinases (Velden and Versnel 1998).

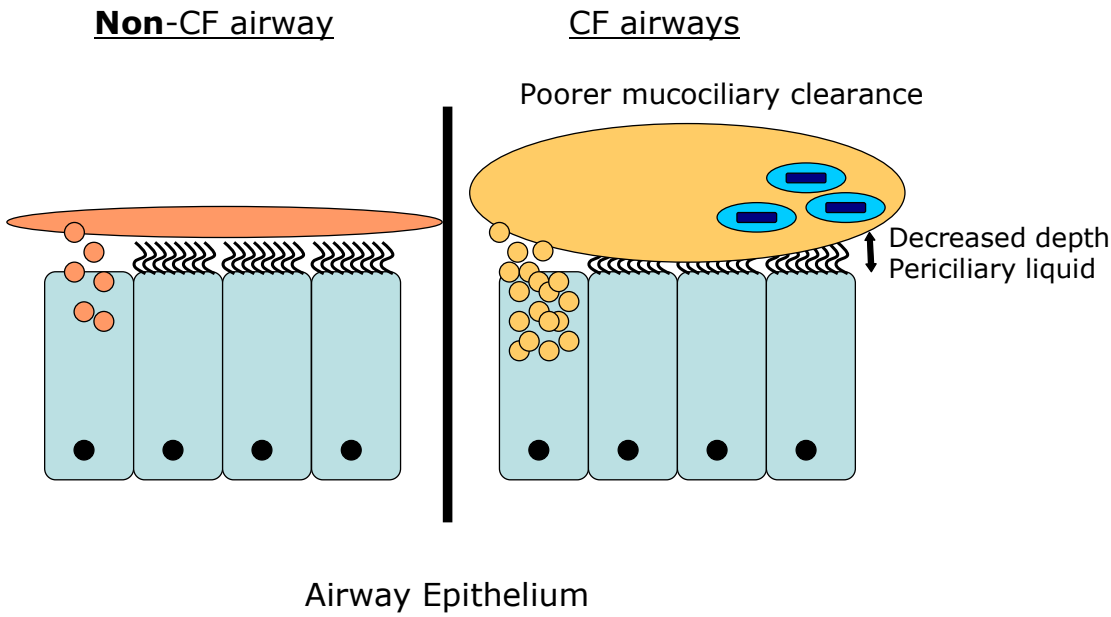

Figure 1.2 Schematic of the airway epithelium in normal (left side) and CF (right side) airways
On the left side the normal human epithelium contains goblet cells, which secrete mucus and ciliated cells, which are responsible for propelling the thin layer of mucus towards the pharynx.
On the right side the situation as found in CF is shown. Goblet cells hypersecrete mucus, which is additionally dehydrated based on the CFTR mutation. This leads to depletion of the PCL and cilia get entrapped in the vast amount of mucus, which can then not be moved anymore by the cilia. This CF lung environment invites bacteria to colonise, leading eventually to chronic infection of CF lungs.

1.1.4.2 Airway Mucus

Airway mucus is a heterogeneous mixture of mucins (0.5-1 %), which are highly glycosylated glycoproteins, salts and dialyzable components (0.5-1 %), secreted polypeptides, water (95-98%), lipids, cells and cellular debris that appear in the airways (Clunes and Boucher, 2007). Additionally mucus also contains soluble antimicrobial substances, such as lactoferrin, which is an abundant substance of the ASL, proteases, antiproteases, oxidants and antioxidants and antibodies (Singh *et al*., 2002, Wine, 1999). Mucins can have very different physiologic roles and mature mucins are subdivided into two groups: membrane bound and secreted. The secreted mucins are responsible for the viscoelasticity of the extracellular mucus layer in the lumen of the airways. Three mucins are expressed in the airways: MUC2, MUC5AC and MUC5B. MUC5AC is predominantly synthesised by goblet cells in the airway epithelium and MUC5B is synthesised in submucosal glands, whereas MUC2 is barely detectable but can be produced in goblet cells. Furthermore mucins show an extraordinary diversity through the carbohydrate side chains, so that they can literally bind any particle that they contact to protect the airways (Thornton *et al*., 1996, Thornton and Sheehan, 2004, Houtmeyers *et al*., 1999).

1.2 Cystic Fibrosis

Cystic Fibrosis (CF) is the most common lethal inherited disease among the Caucasian population and currently affects approximately 9000 individuals (CF Trust, UK). It is an autosomal recessive disease caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene and affects multiple organs, such as the sweat glands, conducting airways and the nasal epithelium, for example. “Cystic Fibrosis of the pancreas” was first described by Anderson in 1938 as pancreatic lesions and later on Di Sant’Agnese *et al.* (1953) found that children with CF show very high levels of sodium and chloride in their sweat (Di Sant'Agnese *et al*., 1953). Gibson and Cooke used this finding to develop a diagnostic test based on the measurement of high levels of electrolytes in sweat, which is still used nowadays (Gibson and Cooke, 1959).

1.2.1 CFTR mutation and its consequences

The mutation of the CFTR gene can cause either malfunction or absence of the CFTR, which is a cAMP-regulated chloride (Cl^-) ion channel. About 1700 individual mutations (www.genet.sickkids.on.ca/cftr) have been identified and reported in the cystic fibrosis mutation database with Δ-F508 mutation (deletion of phenylalanine at position 508) being responsible for about 66% of all mutations in the CFTR worldwide (Donaldson *et al*., 2006). The consequence of any of these mutations are multi-organ disorders as they affect the functioning of lungs,

pancreas, gastrointestinal tract, liver, sweat glands and male reproductive ducts (Wine, 1995) but the severities differ from mild to very serious.

The CFTR ion channel provides a pathway for Cl^--ion movement across epithelium and regulates the Cl^--ion flow, thus it is essential for salt transport, fluid flow and maintaining the essential ionic homeostasis needed for regular functionality, especially in the airways. In normal airways there is a balance between Na^+ ion absorption and Cl- secretion. This balance is regulated by CFTR, which is located in apical membrane of epithelial cells and by another ion channel called ENaC, which is also located in the apical membrane, and which is normally downregulated by CFTR (Boucher, 2007). This transport balance is crucial for the homeostasis of air-surface liquid, which consists of two layers, a mucus layer and a periciliary liquid layer (PCL) as described above (Gail and Lenfant, 1983).

In cystic fibrosis, however, this is a different picture. The mutation of CFTR and therefore absence or malfunction of the Cl^- ion channel leads to uncontrolled Na^+ hyperabsorption as ENaC is not regulated anymore as well as to decreased Cl^- secretion across the apical cell membrane into the cells. Consequently water follows into the cells, which leads to dehydration of ASL and depletion of PCL, which in turn leads to direct interactions of the mucus layer with epithelial cell surfaces (Knowles and Boucher, 2002). The mucus layer contains two major gel-forming mucins in the airways, which are called MUC5AC and MUC5B (Thornton *et al*., 1996) and which upon PCL depletion are able to interact with other membrane-associated mucins, such as MUC1 and MUC4 (Voynow and Rubin, 2009). Furthermore epithelial cilia get trapped and are not able to propel the mucus and all entrapped particles and especially bacteria towards the pharynx anymore. This exposes the lung to a vast amount of potentially harmful agents and bacteria but it does not stop here, as in CF it has been reported that mucins are hypersecreted (Welsh and Smith, 1995), as for example the MUC5AC gene is upregulated by bacterial byproducts, such as LPS (Bautista *et al*., 2009). All together this invites bacteria to colonise the airways and finally leads to chronic bacterial infections of CF lungs. This chronic bacterial infection and host inflammatory responses eventually lead to lung obstruction and eventually to patient morbidity.

1.3 Bacterial infections in CF

Lungs of babies that have CF have been found to be structurally normal at birth but following bacterial infections, high levels of mucus are secreted (Girod *et al*., 1992). Pulmonary infections in CF have been reported to involve relatively few species of bacteria, which have been isolated from CF patients. In early stages of CF *Staphylococcus aureus* (*S. aureus*), for example, is the most common pathogen in children and in early adolescents. Later on *S. aureus* is replaced or at least dominated by *P. aeruginosa,* which is the most common pathogen (80%) isolated from adult CF lungs. Another bacterium, which has gained a lot of attention throughout the last 20 years in accordance with it being isolated more often, is *B. cepacia ,* which is known to be transmissible between patients and usually leads to a rapid decline in lung function (Burns *et al*., 1998, Govan *et al*., 1993).

Most bacterial infections are localised in the larger and smaller airways rather than in the alveoli. In general all bacterial infections start with the adherence of the pathogen to the host cell surfaces, to the mucus layer provided in CF airways or to extracellular matrix (ECM) using different adhesins (Ulrich *et al*., 1998). *S. aureus* is known to cause chronic endobronchial infections in CF lungs and has been shown to preferably adhere to mucus in the airways, for example (Ulrich *et al*., 1998). The focus in the second part of this study will be directed towards *S. aureus, B. cepacia* and *P. aeruginosa*.

1.3.1 *Staphylococcus aureus* (*S. aureus*)

S. aureus is a gram-positive bacterium that is consistently isolated from CF patients but considering the high prevalence, *S. aureus* infections in the airways are still ill defined in terms of how this bacterium successfully initiates and establishes infection in CF. Before there were antibiotic treatments available for this organism *S. aureus* was regarded as one of the main colonising bacteria in CF and was considered lethal for children with CF and contributes to patients poor prognosis and mortality in adults. Nowadays there are quite successful antistaphylococcal and prophylactic antibiotics available (Flucloxacillin), which keep this organism at bay and *S. aureus* is not usually considered to be the common cause for morbidity and mortality in CF anymore. Prophylactic antibiotics have been considered to lead to early acquisition with *P. aeruginosa* though (Elborn, 1999). *S. aureus* and other bacteria cause an inflammatory response, which can lead to irreversible lung damage in CF (Armstrong *et al*., 1995). The repertoire of virulence factors is big and numerous components of *S. aureus* cell membrane, such as lipoteichoic acid (LTA), peptidoglycan and other lipoproteins have been

shown to induce an inflammatory response in epithelial cells (Fournier and Philpott, 2005). In case of the pattern-associated molecular pattern (PAMP) LTA, it has been show that the pattern recognition receptor (PRR) toll-like receptor (TLR) 2 is necessary for successful induction of a pro-inflammatory response and cytokine/chemokine secretion, for example (Greene *et al*., 2005b). Additionally there are other receptors presented on epithelial cells that can interact with *S. aureus*, such as asialoGM1, which leads to IL-8 expression and leading to recruitment of neutrophils (Normark *et al*., 2004). Protein A is another important adhesin and immunostimmulatory component of *S. aureus* that is able to directly interact with tumor necrosis factor α (TNF-α) receptor 1 (TNFR1) presented on most airway cells and induce inflammation (Gomez *et al*., 2004). Furthermore *S. aureus* secretes different virulence factors but the exact roles they play in CF have not been fully determined yet.

1.3.2 *Burkholderia cenocepacia* (*B. cepacia*)

Another bacterium that has become more prevalent in CF is the gram-negative and motile B. cenocepacia (in this work referred to as *B. cepacia*), which was first described by Burkholder (1949) as a phytopathogen causing soft rot of onion bulb.

As for *S. aureus* the first step in infection is adhesion to the airway lumen. It has been shown that this bacterium adheres through proteins and glycolipid (asialoGM1) receptors but also to secretory mucins (Sajjan and Forstner, 1992). Binding to mucins has been shown to occur only when the bacterium expresses the cable pilus and it has recently been reported that through this the bacterium can bind to epithelial cells and induce inflammation (Sajjan *et al*., 2000a). *B. cepacia* is also able to bind to TNFR1, similar to *S. aureus* and induce a pro-inflammatory response via this receptor (Sajjan *et al*., 2008). Furthermore there are also secreted virulence factors and cell wall components, such as LPS. This virulence factor plays a dual role in case of *B. cepacia* as this contributes to the high bacterial resistance to antimicrobial peptides and it is a potent inducer of pro-inflammatory responses (Bamford *et al*., 2007). In CF it has been reported that *B. cepacia* causes infections in only 3.5 % of CF patients in the world but *B. cepacia* infections are usually accompanied by a rapid decline of lung function and poor prognosis (Courtney *et al*., 2004). Furthermore this bacterium has been shown to be transmissible (LiPuma, 2003)and cause the “cepacia syndrome” (fever, pneumonia, bacteraemia) in about 20 % of infected CF patients (Isles *et al*., 1984). *B. cepacia* also secretes various other virulence factors that can cause enhancement of the pro-inflammatory response of the host but many details of *B. cepacia* pathogenesis are not known yet and further research is needed to elucidate the dynamic interactions between the bacteria and the host to develop new strategies and therapies to enhance the quality of life with CF.

1.3.3 *Pseudomonas aeruginosa* (*P. aeruginosa*)

Even though *P. aeruginosa* is non-pathogenic to the normal respiratory tract it is the most common pathogen found in CF, which has been reported to appear in about 80 % of CF patients at the age of 18 and older (CF Trust UK). Chronic infection is now responsible for the large majority of excess morbidity and mortality in these patients, since once infected, it is almost impossible to eradicate this organism, which often displays multi-drug resistances from the respiratory tract (Nguyen *et al*., 2007). Like for the other two bacteria it has been shown that many *P. aeruginosa* products, including lipoproteins, LPS, flagella and other secreted virulence factors contribute to the pathogenesis of these bacteria. The strong inflammatory response produced as result of *P. aeruginosa* infection leads to irreversible lung damage and a decline in lung function eventually leading to morbidity and mortality of the patient (Ratjen and Doring, 2003). *P. aeruginosa* adherence to the airways has also been shown to involve interactions with mucins (Ramphal *et al*., 1991). CF patients are highly susceptible to these infections and even though extensive research has been dedicated to this organism it is still not understood in detail how *P. aeruginosa* persists in CF airways (de Bentzmann *et al*., 1996c). Again different receptors have been identified that take part in delivering the inflammatory response and lead to the production of chemokines and cytokines, for example. As earlier mentioned TLR-2 recognises gram-positive bacteria via LTA and TLR-4 recognises gram-negative via LPS. TLR induced responses usually lead to an activation of NF-κB and the secretion of IL-8 and other cytokines depending on the signalling pathway (Greene *et al*., 2005b, Hauber *et al*., 2005). *P. aeruginosa* also has an additional repertoire of virulence factors that support this persistent infection in CF patients, such as elastase for example. This virulence factor has been shown to rapidly decrease epithelial cell layer integrity and traverse the epithelium, leaving tissue damage (Azghani, 1996). These are only a few ways of *P. aeruginosa*'s virulence and ability to evoke a pro-inflammatory response but points out the importance of understanding the pathogen-host interaction to be able to develop new therapies and to increase the quality of life for CF patients.

1.4 Lipopolysaccharide (LPS)

LPS was discovered at the end of the nineteenth century by Richard Pfeiffer (Westphal, 1977) and was later described as a heat stable and cell-associated macromolecule, which is found on the outer membrane of gram-negative bacteria. LPS can be shed by bacteria during growth, exposure to antibiotics and death. LPS is composed of three distinct structural components. The O-antigen, the core region, which is subdivided into inner and outer core and the Lipid A region, which is the endotoxic unit of LPS (Galanos and Freudenberg, 1993). The O-specific chain enables

bacteria to survive *in vivo* as it makes the bacteria resistant to phagocytosis and killing by complement of the host's immune system (Poxton, 1995)(figure 1.3).

Figure 1.3 Schematic of LPS structure (Anon, 2011); LPS is the major cell wall component of Gram negative bacteria and is composed of three main subunits. The first subunit is a repeating sugar unit (green hexamers) called the O-antigen, the second unit is the core oligosaccharide (inner and outer core) and the last unit, which anchors LPS in the cell wall and makes it immunostimulatory is a region called Lipid A.

LPS of gram-negative bacteria has been shown to be able to induce an inflammatory host response and is continuously present in chronic infected CF lungs. This host response is initiated with LPS binding to a glycoprotein called LPS-binding protein, which is synthesized in the liver and present in plasma. LBP binds LPS, upon which this complex is transferred to CD14. This glycoprotein accelerates the transfer but it is not necessary for the binding to CD14 (Hailman *et al*., 1994). CD14 is located on host cells, such as monocytes, and it does not have an intracellular domain. This is the reason that another receptor (PRR) is involved it transmitting the signal to the inside of the cell. This receptor is TLR-4, which builds a complex with MD-2 and therefore plays a crucial role in innate immunity as this causes a rapid inflammatory response including the secretion of IL-8 (Greene *et al*., 2005b). TLR-4 has an intracellular domain that then recruits other proteins involved in this pathway to translocate NF-κB from the cytoplasm to the nucleus, where this complex then can activate the transcription of target genes, such as IL-8 (Kim *et al*., 2005a).

1.5 Available therapies for CF patients

Several treatments for CF patients are available that all aim to increase the quality of life and expand the life expectancies. The age given for the median predicted life survival for someone with CF is about 35 but it needs to be always kept in mind that different people with different mutations are differently affected by this multi-organ disease.

Helping patients to loosen the mucus is one major objective of several everyday routines that are carried out by patients, which sometimes require help. There are airway clearing techniques,

such as chest physical therapies and postural drainage. Furthermore there are devices that help removing mucus in different ways, such as through oscillation of the airways. There is oscillating positive expiratory pressure, for example, where the patient breathes out through a device, which causes the large and the small airways to vibrate and therefore partially loosens the mucus, which can eventually be coughed up. There are other breathing techniques, which all help to get the mucus moving and these can all be adjusted to the patients needs. Furthermore there are more commonly known little helpers, such as Ibuprofen and hypertonic saline and in addition there are certain recommendations for patients in terms of nutrition but these are all add-on therapies and usually other therapies are required (http://www.cff.org/treatments).

There are several antibiotics commonly used in CF but the therapeutic management of chronic infections with *P. aeruginosa* for example remains challenging. One macrolide antibiotic that was originally used in patients with panbronchiolitis infected with *P. aeruginosa* looked like a potential therapy for CF and a randomised controlled trial was carried out at the beginning of this century. Patients that were included were six years and older and had to have a *P. aeruginosa* infection for one year or longer. After the trial it was reported that Azithromycin treatment was associated with improvement of lung function (FEV_1), less exacerbations and weight was gained by the patients compared to the placebo group. Patients of the azithromycin group also reported that they noticed improvements in physical functioning. (Saiman *et al*., 2003). After this trial azithromycin was observed for its anti-virulence effects and it was shown that there is a correlation between *in vitro* anti-virulence effects (*P. aeruginosa* isolates) and the improvement of CF patients (Nguyen *et al*., 2007). Azithromycin has antibacterial effects but is not bactericidal or bacteriostatic against *P. aeruginosa* at the concentrations realistically achievable in sputum (Baumann *et al*., 2004) but it has been shown that macrolides can decrease the production of virulence factors, such as elastase (Kita *et al*., 1991). This decrease in virulence factors possibly has a direct influence on the pro-inflammatory effects of *P. aeruginosa*.

A new antibiotic has been launched in 2010 in the USA, called Cayston (Aztreonam Lysine), which is an antibiotic to inhale and is the first new developed one of this kind in more than a decade. Cayston is an aerolised formulation of the monobactam antibiotic aztreonam and lysine (Gibson *et al*., 2006). This antibiotic acts against aerobic gram-negative bacteria and was shown to improve lung function of CF patients and reduce the bacterial density in sputum in clinical trials (McCoy *et al*., 2008, Plosker, 2010). These outcomes show that this antibiotic is an addition to the therapies available for chronic infections in CF. These are just a couple of examples of what is available and usually these conventional therapies are used in combination or alteration with others to achieve the most beneficial outcome for every patient. The maintenance and care

is one side of the story and then there is the situation, where a patient has got severe lung disease and all other conventional treatments do not help anymore that is when a lung transplantation is considered. Roughly 120-150 transplants are carried out each year in the USA but the general problem of organ shortage is also present here. In 2003, 368 CF patients were accepted for lung transplant but 524 applied. An issue that was raised in terms of organ shortage for transplantation is the low number of living donors, who are willing to give one of their lobes. If two people do this one CF patient can have a new lung. This whole process is obviously more complex in the way that donors must match the recipient in biological features, such as blood type (www.cff.org).

Despite all these and other treatment options 90 % of CF patients will eventually die of respiratory failure.

1.6 Gene therapy for CF

The main aim of gene therapy for CF is to deliver the CFTR gene efficiently to target cells to cure or slow down the progression of disease. The excitement about gene therapy was big once the CFTR gene was discovered in 1989 and it still is but it has been trial and error for the last 20 years. More than 20 clinical trials were carried out so far using viral and non-viral gene transfer agents. There are some problems that were encountered on the way and which lead to a reasonable slow progress in this field of research (Davies and Alton, 2010).

Problems that were encountered in different trials include efficiency of delivery, host immune responses, the determination of efficacy itself and it is uncertain what degree of delivery need to be reached for clinical benefits. Soon after the discovery of the CFTR gene researchers isolated CF cells from a patients and successfully delivered wild-type CFTR DNA (Drumm *et al*., 1990), which was also repeated in CF mice using liposome-mediated gene delivery (Alton *et al*., 1993). These initial attempts were soon followed by studies in humans using either viral or non-viral gene-transfer.

The viral delivery systems that were used to date include engineered adenovirus or adeno-associated viruses but again problems were emerging, including absence of receptors expressed on epithelial cell surfaces (Walters *et al*., 1999), the induction of inflammatory responses (Crystal *et al*., 1994) and in case of repeated application the immune response decreased the expression rate dramatically, probably by neutralizing antibodies synthesised in the host (Zabner *et al*., 1996). Non-viral delivery systems include cationic lipids (Caplen *et al*., 1995), compacted DNA nanoparticles (Konstan *et al*., 2004) and naked DNA (Zabner *et al*., 1997, Ruiz *et al*., 2001). The main advantage that has been shown for these non-viral systems is the possibility of repeated

administration but some of these also elicit side effects in CF patients. Side effects have not been reported for every trial and if present they can vary from very mild flu like symptoms (Zuckerman *et al*., 1999) to unacceptable toxicity (Crystal *et al*., 1994). In one trial initial assessment of an adeno-associated virus transfer and functional CFTR expression was found quite successful as lung function improved in accordance with higher levels of IL-10 and lower levels of IL-8 (Moss *et al*., 2004) and therefore a larger scale trial was carried out, in which these clinical improvements were not observed (Moss *et al*., 2007). Non-viral systems were used mainly in the form of lipid-based gene-transfer and also resulted in variable outcomes. The efficacy has generally been found variable but one trial has reported success in terms of repeated administration to the nasal epithelium and no loss of efficacy (Hyde *et al*., 2000). However, delivery of CFTR to the nose as indicator of safety has been criticised as in another trial it was also found that in case of cationic-lipid mediated CFTR delivery to the nose, no symptoms were seen but when delivered to the lung there were CF patients experiencing mild-influenza like symptoms (Alton *et al*., 1999).

It has been shown over the years that CFTR can safely be delivered to the airways and even repeatedly when non-viral systems are used. Repeated delivery to the airways is an essential feature as the there is a constant turnover of the epithelium present. In contrast the expression level of CFTR declined quickly and side effects were observed for both delivery systems, plus it is not known how efficacy will correlate with clinical benefit. The main focus of the UK Cystic Fibrosis Gene Therapy consortium is directed to develop a non-viral delivery system that can be administered repeatedly and that leads to clinical benefits for the patient. Alongside the aim is to increase the period of expression of CFTR. One system was assessed and found to meet certain criteria, such as nebulizable, repeatable and to have a good preclinical toxicity profile. The cationic lipid GL67, which was used in the trial reported by Alton *et al*. (1999), was identified to fulfil these requirements and is currently being modified to reduce the side effects found after delivery to the airways and to improve gene expression duration (Davies and Alton, 2010). Gene therapy needs further improvements to verify safety and efficacy but also further understanding of correlation between changes of the CFTR expression in patients and what they identify as clinical improvements to lead to a successful treatment that can either slow down, halt or cure CF lung disease.

1.7 Modelling the disease

1.7.1 Animal models

Animal models have been used in research for several different approaches, not just CF but also COPD, asthma and for other non respiratory research fields. For CF several animal models have been developed throughout the years and the main organisms that have been used are mice. Although there are many other animal models, including rat, guineapig and the novel pig and ferret models, these are all *in vivo* systems that have mostly been genetically modified in the attempt to create an animal that shows CF pathogenesis in several, ideally all CF affected organs. This overall aim has yet to be achieved though. Next to *in vivo* models there are several *in vitro* models employing usually one cell line or primary cells. A couple of co-culture *in vitro* models have been developed and some will be discussed here further on. Here it is the aim to give a short insight into the *in vivo* and *in vitro* models that are available for CF airway research.

1.7.1.1 Rodent models for CF research

Several mouse models have been developed up until today for further investigation of the development of CF lung disease and a quick look at the history of these models will point out where they stand today.

At the beginning the models were not always genetically modified, such as models for chronic lung infection, which is also a hallmark of CF. One of the first models for chronic bronchopulmonary infection was a rat model, which was infected with *P. aeruginosa* bacteria, which were embedded in agar beads to retain the bacteria in the airways and mimic a chronic infection (Cash *et al*., 1979). There were similar models to this using agarose beads instead (Klinger *et al*., 1983)and these beads changed further down the line to alginate in the attempt to closer mimic CF *P. aeruginosa* infection, where these bacteria grow in a alginate biofilm (Wu *et al*., 2001). This rat model was then adapted to mice to study chronic mucoid *P. aeruginosa* infection and to study bacterial pathogenesis in CF (Starke *et al*., 1987). The genetically modified mouse models where CFTR gene in the mouse DNA has been disrupted by homologous recombination in embryonic stem cells, which enables researchers to target a mutation to a specific site in the chromosome (Dorin *et al*., 1992, Snouwaert *et al*., 1992, Thomas and Capecchi, 1987). Furthermore in other mice the human known ΔF508 mutation (and others) was introduced into the homologous mouse gene loci to generate CF mice (Zeiher *et al*., 1995). One problem with these animal models that became obvious quite quickly was the predominant intestinal pathogenesis and in contrast these mice did not show any sign of lung disease, which is

the main cause of morbidity and mortality in humans. Additionally recombinant CF mouse models were developed with clinically relevant mutations, which were introduced to the mouse gene, such as earlier mentioned ΔF508 (Zeiher *et al*., 1995) or G551D mutation (Delaney *et al*., 1996). After all generating all these mouse models, which have been fundamental for CF research, transgenic KO mice were developed, which allowed preclinical testing of mutation-specific treatment approaches (Du *et al*., 2002). However, for all these generated models it important to keep in mind how they were generated and that this could have an effect on possible phenotypes and therefore analysis results, which need to be taken into account.

So what about lung disease? The phenotypes of the previously mentioned mice models did not show any signs of spontaneous lung disease and also there were ground breaking differences in the phenotypes. In CFTR KO mice, whether a complete KO or with some residual CFTR, the intestinal disease is the most prominent feature in accordance with decreased body weight and mucus plugging of intestine leading to death without showing a development of lung disease because of chronic inflammation and bacterial infections as it is seen in human CF. Even though some models were reported to be less able to fight infection and then showed increased inflammation, especially after repeated bacterial challenges, lung disease as seen in humans was not observed (Davidson *et al*., 1995). There are several things to be aware of using animal models and to interpret findings in certain phenotypes in the attempt to understand the human disease as mouse models show different CF severities in phenotypes depending on the background of the mouse, the mutation and the expression levels of CFTR. In terms of lung pathology there was little evidence of changes, which has been suggested to be due to the sterile facilities most mice are kept in and that the changes that are seen in human lungs occur over years and not weeks (Kukavica-Ibrulj and Levesque, 2008). Then finally in 2004 Mall *et al*. (2004) presented the first mouse models that actually displayed lung pathology similar to what is found in humans. In this mouse model not CFTR was modified but the sodium channel β-ENaC was overexpressed and caused hyperabsorption of sodium ions, leading to depletion of ASL, dehydrated mucus and these mice showed characteristics of lung disease. This early disease state was followed by neutrophillic inflammation and increase in pro-inflammatory cytokines in airway liquids. Upon bacterial challenges these mice were unable to clear the infection. The outcome here showed that ion flow directly influences the height of the periciliary liquid (PCL), that ion flow alteration can lead to CF pathogenesis in the small airways and that inflammatory response is initiated by dysfunction of ion flow and PCL depletion (Mall *et al*., 2004).

In addition to these mouse models there are others but only two recent once will be introduced here. After the development of gene targeting other animal models were generated, such as pigs and ferrets. On one hand these animal models have the advantage of being bigger and easier to

analyse and on the other hand lung anatomy is more similar than mouse lungs compared to what is seen in humans.

1.7.1.2 The new pig model for CF research

In 2008 Rogers *et al*. (2008) reported the first time about the "successful" development of CF piglets.

The researchers disrupted the CFTR gene in fibroblasts of normal pigs by homologous recombination and somatic cell nuclear transfer to generate mixed ($CFTR^{-/-}$,$CFTR^{-/+}$ and $CFTR^{+/+}$) litters of pigs. At birth $CFTR^{-/-}$ piglets appeared normal and apart from genetic analysis could not be identified to be different from the other litter mates but whereas meconiul ileus in humans only appears at a rate of 16 – 20 % (Dorfman *et al*., 2009) it was 100 % for the CF pigs, which had to be surgically solved for survival of the pigs. Other CF symptoms found included pancreatic destruction, abnormalities of gallbladder and the normal appearance of lungs at birth but again the disease pathological appearance in detail is different (Rogers *et al*., 2008). In these CF piglets the lungs at birth were normal compared to the non-CF litter mates and did not show increased inflammation. BAL was analysed only 6-12 hours after birth and no increase neutrophil count or other signals of inflammation were found but x-ray tomography revealed progressive thickening of airway walls and scattered infiltrates, similar to humans. Further analysis of BAL showed that CF pigs more often had bacteria present in the airways and at higher concentrations, including *S. aureus*. It was also reported that CF piglets were unable to efficiently eradicate bacteria and were less frequently sterile when compared to the non-CF piglets (Stoltz *et al*., 2010). This model provides new insights into the development of lung disease, especially straight after birth but researching these pigs takes time as pigs have a much longer life cycle compared to mice.

1.7.1.3 The new ferret model for CF research

Another model that has recently been developed using gene targeting is the CF ferret model and the idea of this being a good CF model was driven by the higher similarity (compared to mouse) of lung anatomy and biology between ferrets and humans and a fast reproduction time of ferrets. After a long struggle to actually get any CF ferrets to survive after birth it was shown that CF ferrets showed pathologies like they are seen in humans. Meconium ileus was observed in 75 % of CF ferrets and a wide variability in intestinal obstruction was seen. Pancreatic disease was reported to be similar as it is in humans. However, the most striking difference of ferrets and humans is the intestinal tract, which lacks a cecum (assists in digestion of plant material). Therefore the weight gain in newborn ferrets was the biggest hurdle and it was suggested that

intestinal CFTR is crucial for absorption of nutrients. Once ferrets survived, a BAL analysis revealed increased bacterial counts one week after birth, which were not accounted for death in these ferrets. More publications are to be awaited and the first conclusion drawn by these researchers was that this model maybe useful for dissecting the pathophysiology found in CF (Sun *et al*., 2010).

All these animal models assist in dissecting the effects of CFTR mutations on the whole organism, however as mentioned above there are species specific differences, which need to be taken into account and care has to be taken to relate results of animal experiments to a human disease like CF. Although animal models have assisted in learning about CF and pathophysiology of infections the limitations of these models must be recognized and great caution must be taken when trying to transfer results to patients.

1.7.2 *In vitro* cell culture models for CF research

Next to all these *in vivo* models there are cell culture based *in vitro* models for CF research, which usually employ either primary epithelial cells or epithelial cell lines that were generated from isolated epithelial cells of healthy or of CF airways. One advantage of these *in vitro* cultures is that they have human origin and therefore species specific differences do not need to be taken into account and therefore *in vitro* cell cultures have been extensively used to investigate airway biology, disease pathophysiology and even the use of novel therapies.

1.7.2.1 Submerged cell culture

The simplest version of cell culture is under submerged conditions in plastic dishes and other tissue culture ware. Submerged cell culture has been used in airway related research but it has been pointed out that these cells do not closely mimic the *in vivo* physiology and morphology. This technique is used to maintain and expand immortalized cell lines in flasks but also it is used for primary cells in terms of maintenance after dissociation of the freshly excised specimen, which can then be expanded before being used for further experiments (Randell *et al*., 2011). As already mentioned, submerged cultures do not necessarily show close similarity to *in vivo* morphology and physiology. One cell line that has been investigated for exactly these differences is Calu-3 and it was shown that under submerged conditions (on a transwell insert rather than plastic) a less suitable model of the tracheobronchial epithelium was established compared to cultures grown at air-liquid interface (ALI) (Grainger *et al*., 2006). There are other reports that compare submerged cultures to ALI cell cultures and they have reported similar

findings in that responses of the two cultures are often different, such as cytokine expression by NHBE cells (Kikuchi *et al*., 2004), or the suscepitibility of primary human nasal cells to *P. aeruginosa* infections (Fleiszig *et al*., 1997). Furthermore it has been emphasised that ALI cultures also show polarized secretion of IL-6 and IL-8 in 16HBE140-, for example (Chow *et al*., 2010). However, some general and very useful laboratory techniques, such as flow cytometry cannot be used for cell cultures grown at ALI because disrupting the differentiated cell layer would possibly cause cell damage and lead to false interpretation of results. They can be used for submerged cell cultures though but whether these results are valuable for comparison to *in vivo* is questionable. There is a need to develop further techniques for characterising and analysing ALI cell cultures. The closest *in vitro* cell culture mimicking the *in vivo* one is achieved using primary cell culture at ALI.

1.7.2.2 Air-liquid interface cell culture of primary cells and transformed cell lines

Primary human airway epithelial cell models have been around for over twenty years (Lechner *et al*., 1982) and they provide the closest representation of airway epithelium *in vitro* as they have not been transformed and should have the same genotype and phenotype as cells *in vivo* when cultured at air-liquid interface (Whitcutt *et al*., 1988), which recreates the natural surroundings of these cells. The human lung is lined by epithelial cells, which face the lumen where air is coming in on one side and subepithelial tissue on the other side (Breeze and Wheeldon, 1977). Primary cells that have been freshly isolated from a specimen are first cultured in plastic dishes, in which they are only poorly differentiated and show a squamous phenotype but this step is necessary for expanding and passaging these valuable epithelial cells. As already mentioned culture of airway epithelial cells at ALI (on porous membranes) allows mucociliary differentiation, which is a complex process and has been shown to involve cell-matrix and cell-cell interactions, differentiation of serous cells and the establishment of correct ion flow properties has not been fully elucidated yet. It has been shown though that there is a differentiation-dependant mucin upregulation (MUC5AC, MUC5B) present in primary nasal and bronchial epithelial cells (Bernacki *et al*., 1999). Furthermore retinoic acid has been shown to suppress or reverse squamous metaplasia in culture (Yoon *et al*., 1999). Even though this first steps in this direction were made in the eighties (Wu *et al*., 1985, Yankaskas *et al*., 1985, Wiesel *et al*., 1983) and was constantly improved (Wu *et al*., 1997) there is still much unknown about detailed regulation of this complex process.

Even though primary cells are a good and relevant model for CF research they are not readily available for many laboratories and depending on the research field not many diseased tissue

samples might be available. Furthermore primary cells have a limited replicative lifespan, which makes the use of primary cells expensive (Randell *et al*., 2011).

Other types of cells that have been extensively used are cell lines and they have been proven to be a valuable complement to primary epithelial cell culture. There are different ways of generating cell lines, including transformation to immortalize these with the Simian Virus 40 (SV40) (Zeitlin *et al*., 1991b) but also cancerous cells, which are already immortal, have been used for airway research (Shen *et al*., 1994). Transformation of primary cells to generate cell lines has not always led to success though as many cells do not survive crisis (Buchanan *et al*., 1990, Reddel *et al*., 1988, Gruenert *et al*., 1988), lose the ability to polarize (Gruenert *et al*., 1988) and therefore do not recapitulate the *in vivo* phenotype and even when they do polarize they sometimes lose certain essential epithelial features, such ion flow (Cozens *et al*., 1994). However there are cell lines with typical epithelial cell features that have been successfully used in CF research, as the cell lines used throughout this project (C38, IB3-1 and Calu-3) and many others (Wan *et al*., 2000, Ehrhardt *et al*., 2006, Greene *et al*., 2005b). Additionally cell lines are often more readily available either commercially or through collaborations of different laboratories. As already described above culturing airway cells at ALI is the preferred technique to generate airway related research models that closely mimic the *in vivo* appearance of the epithelium.

1.7.2.3 Mono- and co-culture systems for airway related research

ALI cultures of primary cells and transformed cell lines for airway related research mimic, as described above, the *in vivo* morphology and physiology of airway epithelial cells closer than submerged cell cultures. Mono-cultures have been extensively used in many areas of research focussing on the airways. For CF research there are many cell mono-culture models that all focus on broadening the understanding of CF lung pathophysiology with different objectives, such as inflammatory responses to the infection with bacteria (Kube *et al*., 2001)or bacterial products (LPS) , bacterial adherence to CF and non CF cells and general characterisation studies looking at tight junction properties and paracellular integrity (Nilsson *et al*., 2010), just to name a few.

The initial stages of establishing mono-cultures of primary human bronchial epithelial (HBE) cells at ALI for airway related research were not straight forward. Even though much attention has been directed towards cell growth medium and growth supports, such as collagen IV (Fiedler *et al*., 1991), to establish *in vivo* like culture models, the establishment of ALI using a porous membrane as growth support, has been reported to be a very important inducer of cell

differentiation, of which the complex mechanisms are still not fully elucidated (Gruenert *et al*., 1995, Randell *et al*., 2011) but these mono-culture systems have made a huge difference to research and the collected knowledge of the airways, the function of different cell types in the airways and enabled to further investigate diseases, such as CF. These models made it possible to study biochemical and genetic mechanisms of differentiated epithelial cells of healthy and diseased origin.

One of the driving forces behind developing models is the ability to study ex vivo cell cultures in a simplified setting compared to *in vivo*, to reduce and partially replace animal models. Like the ALI culture models display a simplified version of the airways (epithelium) and are nowadays routinely used in the attempt to answer basic research question, discover molecular mechanism and in terms of CF identify the link between CFTR mutations and lung pathogenesis.

However, *in vivo* there is not only one cell type present, it has long been known that the airways consist of different cells that all play their role in tissue homeostasis through direct cell-cell interactions as well as through autocrine and paracrine communication via secreted growth factors and cytokines, for example (Breeze and Wheeldon, 1977, Burgel and Nadel, 2004, Knight, 2001). Epithelial cell interactions with subepithelial fibroblasts have been reported to be important as this modulates cell behaviour, proliferation and differentiation of the epithelium and vice versa (Minoo and King, 1994). For example it is known that epithelial wound repair is dependent on their own actions, their interactions with the ECM and on the cytokine milieu, which is established by themselves but also other surrounding cells in the airways, such as bronchial wall fibroblasts, which secrete cytokines and modulate epithelial cell function (Thompson *et al*., 1995). Compared to CF research more attention has been paid to subepithelial fibroblasts in asthma research and certain growth factors and interleukins (IL) have been detected in airways, epithelial cells as well as fibroblasts, including IL-8, IL-6, hepatocyte growth factor (HGF) and several members of fibroblast growth factors (FGF) (Holgate *et al*., 2000, Knight, 2001, Sacco *et al*., 2004).

Even though more information has been gathered about factors that modulate epithelial cell proliferation and differentiation, the mechanisms are not completely known yet. To further elucidate underlying mechanisms of these interactions between epithelial cells and fibroblasts, possible growth factors and cytokines, for example, a few co-culture systems have been developed so far. Understanding the mechanisms of communication may lead to novel targets for therapies for respiratory diseases.

One of these co-culture models was established by Skibinski *et al*. (2007) to look at the role of HGF in terms of bronchial epithelial growth regulation. In this study as well as in most others fibroblasts were used as feeder layers, which have been treated with mitomycin C to inhibit

proliferation and inhibit possible overgrowth of fibroblasts, which were grown in the bottom of a 24-well plate, not allowing direct cell contact to epithelial cells grown on TWs. This group showed that HGF has growth promoting effects on bronchial epithelial cells, which is in accordance with other reports (Thompson *et al*., 1995, Skibinski *et al*., 2007). Additionally it was pointed out that HGF is important but not the only stimulating compound secreted by fibroblasts, as the inhibition of HGF did not lead to complete proliferation inhibition of bronchial epithelial cells. Furthermore it was shown that the inhibition of TNF-α and IL-6 using specific antibodies reduced the co-culture HGF production and therefore epithelial cell proliferation. These findings emphasize that there is a whole communication network to be discovered for epithelial cells and subepithelial fibroblasts. An additional study further emphasises this as Myerburg *et al*. (2007) have shown that subepithelial fibroblasts establish a suitable environment for human bronchial epithelial cell differentiation. It has been shown here that subepithelial fibroblasts, which were actively proliferating on the undersurface of the TWs, increased ciliogenesis, for example (Myerburg *et al*., 2007).

These are two examples of epithelial cell and subepithelial fibroblast co-cultures that clearly show and emphasise that these intercellular interactions and communications via secreted factors are important and further investigation is needed to elucidate the role of subepithelial fibroblasts and direct cell-cell interactions in CF lung disease.

1.8 Aims of this study

The overall aims of this study were to design a novel co-culture model of normal and CF human airways *in vitro* (figure 1.4)

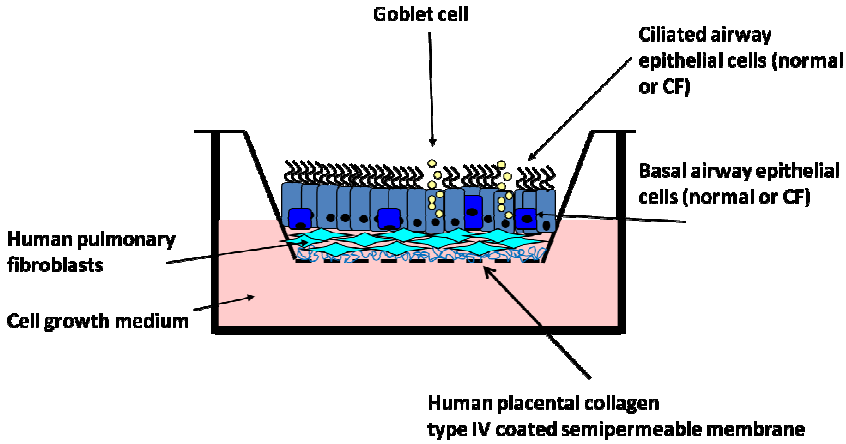

Figure 1.4 Schematic of the established co-culture model for normal and CF human airways *in vitro*
The schematic shows one well of a 24-well tissue culture plate with an inserted TW. The porous membrane of this TW was collagen IV coated before human pulmonary fibroblasts were seeded into the TW. After adequate incubation time epithelial cells were seeded on top and kept submerged for 4 days before ALI was established to induce differentiation of the epithelial cells.

The specific aims were therefore:

- To establish mono-culture models of each cell type for characteriation
- To determine a suitable growth medium and growth substrate for the co-culture models to verify normal cell growth of both cell types
- To characterise each mono-culture system in terms of cell type specific markers to be able to localise epithelial cells and fibroblasts in the co-culture system
- Analysis of epithelial cell morphology in mono- and co-cultures to validate fibroblastic influence on epithelial growth and differentiation
- Characterise pro-inflammatory responses, cell viability and TER of mono- and co-cultures following LPS stimulation, following live bacterial challenges or after stimulation with HIA bacteria

The development of non-CF and CF co-culture models, comprising epithelial cells and fibroblasts in close proximity, allowing physical contact, will help to elucidate the cross talk between these two cell types and help to investigate how fibroblasts take part in the inflammatory response to bacterial challenges in CF.

2 Chapter 2 Material and Methods

2.1 General consumables

All reagents were purchased from Sigma (Poole, UK) unless stated otherwise. Cell culture consumables were purchased from Appleton Woods and Greiner Bio-One (Birmingham, UK). Normocin (100 µg/ml end concentration) was bought from InvivoGen, San Diego. Fetal bovine serum gold (FBS), Dulbecco's modified eagle medium: Nutrient mixture 12- HAM (DMEM/F12) growth medium, Phosphate saline Buffer (1x, PBS), L-glutamine and Trypsin-EDTA were purchased from PAA Laboratories Ltd (Sommerset, UK). BD Falcon Transwell® inserts and companion plates were purchased from VWR (Leicestershire, UK). Normal human pulmonary fibroblasts, fibroblast growth medium 2 (HPFM), airway epithelial cell growth medium (AEM) were purchased from Promocell (Heidelberg, Germany). Vectashield hard set mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, UK). Immobilon® Western Enhanced Chemiluminescent substrate (ECL) was purchased from Millipore (Watford, UK), X-ray film and DeStreak solution was purchased from Amersham Biosciences (Buckinghamshire, UK). Mueller-Hinton broth and Mueller-Hinton agar were purchased from OXOID LTD. (Basingstoke, UK). Calu-3 cells were purchased from American Type Culture Collection (ATCC). 4 well microscope slides were purchased from C.A. Hendley (Essex) LTD. (Essex, UK). Phenol, Kodak GBX developer and Kodak GBX fixer and replenisher were purchased from Sigma (Poole, UK). Nunc Maxisorb ELISA plates, Microscope slides (1.0-1.2mm thick) and coverslips (0.13-0.17mm thick) were purchased from Fisher Scientific (Loughborough, UK). Immersol™ Immersion oil was purchased from Carl Zeiss Ltd (Hertfordshire, UK). Petri dishes were purchased from Sarstedt Ltd. (Leicester, UK).

2.2 Antibodies

Mouse monoclonal antibody to fibroblast surface protein (1B10; ab11333, 0.2 mg/ml), mouse monoclonal anti-cytokeratin 8 antibody (0.5 mg/ml, ab9023), mouse monoclonal anti-basal cell cytokeratin (CK5) antibody (1 mg/ml ab9272) and mouse monoclonal anti-mucin 5AC (ab3649, 0.2 mg/ml) antibody were purchased from Abcam (Cambridge, USA). Mouse IgG kappa (Mopc 21, 5 mg, m-7894), and goat anti-mouse fluorescein isothio-cyanate (FITC; F0257) antibodies were purchased from Sigma. The mouse monoclonal anti-vimentin [V9] antibody was purchased from Gene Tex (GTX76575) and mouse monoclonal anti-Zonulae occludens protein 1(ZO-1; 0.5 mg/ml) antibody was purchased from Invitrogen.

2.3 Cell types

There are different cell types available for research use. If cells are taken directly from a living organism (e.g. biopsy) these cells are called primary cells and some primary cells are also commercially available, in this case human pulmonary fibroblasts. When a cell line is established primary cells are manipulated in terms of transformation to alter their properties to an immortal cell line. The third category of cell lines available is cancerous cell lines, which are already immortal due to their cancerous properties. Cell lines do differ slightly to the cells of their origin, as through transformations and long-term *in vitro* culturing gene expression can change.

2.3.1 Primary cells

The only primary cells that were used throughout this project were human pulmonary fibroblasts (HPF), which originated from normal lung tissue and are commercially available from Promocell. As primary cells are taken directly from a living organism (biopsies) without being transformed they should represent the same geno- and phenotype as the same cells *in vivo*, which is an advantage as they will possess the same morphological and biochemical characteristics as *in vivo* but primary cells are in general short lived as they can only be used for a small number of passages, in this case from P2-P7, as recommended by the manufacturer (Promocell).

2.3.2 Transformed and cancerous cell lines

Transformed cell lines originate usually from a specific tissue of a specific species and through transformation they are immortalised, which means they do not die after a couple of passages but keep dividing and growing in cell cultures, potentially giving unlimited supply of these cells.
IB3-1 is a bronchial epithelial cell line that originates from a cystic fibrosis patient. This cell line was immortalized in 1992 by transformation with a hybrid virus, adeno-12-SV40 and all cells are deficient in cyclic-AMP-medicated protein kinase A activation of chloride conductance, which is diagnostic of CF. IB3-1 are heterozygote for the delta F508 mutation and a non-sense mutation (W1282X) In C38 cells, which originate from IB3-1, the CF phenotype was corrected by transfection with wild-type adeno-associated viral CFTR (AAVCFTR) and the cells stably express wild-type CFTR (Zeitlin *et al.*, 1991a, Flotte *et al*., 1993)
Calu-3 is a cancerous cell line and originated from a lung adenocarcinoma. 12 different cell lines from carcinomas were screened and only 3 of these showed a measurable TER, plus Calu-3 were

the only cells expressing CFTR. Compared to transformed cell lines Calu-3 did not undergo crisis and will contain the same properties over repeated passages, resembling serous gland cells (Shen *et al*., 1994)

2.4 Cell culture

2.4.1 Human placental collagen type IV as growth substrate

Human placental collagen type IV (Sigma, collagen IV) is acid soluble and was dissolved in 3 % sterile filtered acetic acid, as per manufacturer's instructions. All cell culture materials such as cell culture flasks, Transwell® inserts and 4-well slides were coated with collagen type IV. The volume of human placental collagen type IV used for each device is dependent on the surface area as the final concentration to be worked with is 10 µg/cm^2. Once the collagen was applied all devices were incubated at room temperature for 1 hour before the excess human placental collagen type IV was aspirated and devices were washed three times with 1 x PBS. Human type IV collagen acts as a substitute basement membrane for cells *in vitro* and encourages them to proliferate and spread. This mimics the *in vivo* physiology closer than using plastic tissue culture devices (Fiedler *et al.*, 1991). Collagen coated devices were stored at 4° C for up to one month if not used immediately for culturing cells and experiments.

2.4.2 Culturing cells from frozen

Frozen vials of cells were removed from liquid nitrogen and defrosted quickly in a water bath at 37 °C. Once thawed, the 1 ml cell suspension was transferred to 5ml warm cell specific culture medium placed in a 15 ml Falcon tube. The cell suspension was then spun down at 250 x g for 5 min. The resulting cell pellet was resuspended in 10 ml of cell culture medium and directly transferred into a collagen type IV-coated 75 cm^2 tissue culture flasks. The cells were then placed in an incubator at 37°C, 5 % CO_2 atmosphere after labelling the 75 cm^2 tissue culture flasks with name of user, cell type, and date and passage number.

2.4.3 Cryopreservation of cells

Cell growth was monitored using a light microscope and at 80-90 % confluency, cells were washed with PBS to remove FBS, which would inhibit the activity of trypsin. For 75 cm^2 culture flasks, 2.5 ml of trypsin/EDTA (0.25 %) was used to lift the adherent cells off the culture device. Once trypsin-EDTA was added to the culture flasks these were returned to the cell culture incubator at 37 $^{\circ}C$, as this is the optimum temperature for enzyme activity of trypsin. Once the cells were detached from the flask, cell culture medium was added (5 ml) to inhibit the activity of trypsin and then cells were transferred to a falcon tube to be spun down at 250 x g for 5 min. Supernatant was discarded and the cell pellet was resuspended in freezing solution, which contains 10 % (v/v) dimethyl sulphoxide (DMSO), 40 % (v/v) cell specific culture medium and 50 % (v/v) (FBS). Cells from one 75 cm^2 culture flasks were resuspended in 3 ml freezing solution and then 1ml was transferred to each of three labelled cryotubes. The vial was then put into a "Mr Frosty" freezing container containing 250 ml isopropanol that allows cell to cool at a rate of -1°C/min. The freezing container was placed in a -80 °C freezer for 24 h before the vials were transferred into the liquid Nitrogen cell bank.

2.4.4 Submerged cell culture

Human pulmonary fibroblasts (HPF) were maintained in fibroblast growth medium 2 containing 12 % FBS (unless otherwise stated), normocin (50mg/ml), 2mM L-glutamine and the manufacturer's supplement (full HPFM). The supplement consisted of FBS (0.02 ml/ml), basic fibroblast growth factor (recombinant human, 1 ng/ml) and insulin (recombinant human, 5 µg/ml).

C38 and IB3-1 cells were maintained in full medium comprising Promocell® airway epithelial growth medium (AEM) supplemented with 5 % FBS (unless otherwise stated), normocin (50 mg/ml) and the manufacturer's supplement (full AEM). This consisted of bovine pituitary extract (0.004 µl/ml), epidermal growth factor (recombinant human, 10 ng/ml), insulin (recombinant human, 5 µg/ml), Hydrocortisone (0.5 µg/ml), epinephrine (0.5 µg/ml), triiodo-L-thyronine (6.7 ng/ml), transferrin, holo (human, 10 µg/ml) and retinoic acid (0.1 ng/ml).

Calu-3 cells were maintained in DMEM/Ham's F12, which was supplemented with 15 % FBS (unless otherwise stated), normocin (50 mg/ml) and 2 mM L-glutamine.

All cells were cultured in collagen IV coated (10 $\mu g/cm^2$) 75 cm^2 tissue culture flasks under standard cell culture conditions at 37 °C and 5 % CO_2 until 90-95 % confluent. Cells morphology, growth, the degree of confluence and confirmation of absence of any bacterial or fungal

contamination was assessed using an inverted microscope. When cells were 90 % confluent the medium was removed and cells were washed and trypsinised as described in 2.4.3. Cells were then counted and seeded for further experiments or for expansion into more 75 cm^2 tissue culture flasks.

2.4.5 Determination of cell counts

Cell counts were carried out prior to every experiment in order to seed the correct number of cells required for each experiment. After the cells have been trypsinised, spun down and resuspended in fresh cell culture medium, a volume of 10 µl cell suspension was applied to an improved Neubauers haemocytometer. Cells in the four quadrants were counted and the average calculated to obtain the final number of cells per ml multiplied by 10^4.

2.4.6 Cell culture on Transwell® Inserts (TWs)

The cell culture TWs used had a polyethylene terephthalate (PET) track-etched membrane with a pore size of 0.4 µm, pore density of 2 ± 0.2 x $10^6/cm^2$ and an effective growth area of 0.33cm^2. These inserts were aseptically handled with sterile forceps and 12 TWs were placed into a 24-well cell culture insert companion plate before collagen IV was applied to the inserts as described in 2.4.1

For mono-cultures of HPF, C38, IB3-1 and Calu-3, cells were seeded apically onto human placental type IV collagen coated TWs at a cell density of 3 x10^4 cells/well. HPF cells were seeded in HPFM, C38 and IB3-1 cells were seeded in AEM, whilst Calu-3 cells were seeded in full DMEM/F12. Initially all cells were cultured under submerged conditions with 300 µl medium in the apical compartment and 600 µl medium was added to the basolateral compartment.

Four days after seeding cells, the apical medium on the epithelial cells was removed (the epithelial cells were now said to be at air-liquid interface- ALI) and the apical medium on HPF was refreshed. Basolateral medium was refreshed on those same days for all cells. The established air-liquid interface (ALI) induces mucociliary epithelial differentiation. The basolateral medium and any apical liquid from epithelial cultures were then collected every 3 - 4 days and fresh medium was added to the basolateral compartment, whereas for HPF the medium in the apical compartment was also refreshed. All medium collected was frozen at - 80 $^{\circ}C$ until further analysis.

For co-cultures HPF cells were seeded exactly as described above and were cultured under submerged conditions for 4 days prior to removal of the apical HPFM, which was then replaced with epithelial cell suspension. C38 and IB3-1 cells were seeded on top of the HPF cells at the same density as Calu-3 (5 x 10^4 cells/well) but each in their specific medium, either AEM or

DMEM/F12. At this point the basolateral medium was changed to the appropriate epithelial medium for the epithelial cells used in the co-culture. These cultures were then kept under submerged conditions for another four days to allow the epithelial cells to form a confluent and adherent layer on top of the HPF-populated collagen IV basement membrane. Afterwards the medium was aspirated to establish an ALI and induce differentiation of the epithelial cells. Again the basolateral medium and any apical medium were collected every 3 - 4 days and fresh medium was added to the basolateral compartment. This procedure was repeated every 3 - 4 days for a period of at least 14 days. Alongside with the medium change the Transepithelial electrical resistance (TER) was measured as described in 2.9. All samples collected, were frozen at -80 °C until further analysis.

2.5 Cell viability assay - Cell Titer Blue™ (Promega)

Cell Titer Blue (CTB) is an endpoint assay that provides a homogeneous, fluorometric method to monitor cell viability. Viable cells, which are metabolically active, can convert resazurin (blue with little intrinsic fluorescence activity) into its highly fluorescent product, called resorufin (pink). CTB was directly applied to the cell surface, in the cell medium and required an incubation of 2 h at 37 °C prior to analysis (as per the manufacturer's instructions). The fluorescent intensity was measured on a standard multiwell fluorescent plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with a 560 nm excitation, and 590 nm emission wavelength. CTB was used in different experimental layouts, which will be discussed individually in the chapters affected.

2.6 Flowcytometry

Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, USA). This excitation source was an air cooled argon laser emitting a 488 nm beam at 15 mW.

2.6.1 Cell cycle analysis using flow cytometry

The method used for Cell cycle analysis to distinguish cells in different phases of the cell cycle was first described by Nicoletti *et al*. (Nicoletti *et al*., 1991) and was proven to be rapid, easy and reproducible method for monitoring cell cycle changes under certain conditions with special

focus on apoptosis, a controlled mechanism of cell death. Apoptotic cells show distinct morphological changes and physical properties (cell shrinkage and condensation of chromatin for example).

Cellular DNA can be stained using propidium iodide (PI), which intercalates with DNA and emits fluorescence which correlates with the amount of DNA in each cell.

Cells were seeded in their specific growth medium on 24-well cell culture plates at a density of 1 x 10^5 cells/well and incubated overnight at 37 °C, before their own specific medium was refreshed, kept, (this is referred to as conditioned medium) or replaced with fresh or conditioned medium of the other cell types. After a further incubation period of 24 h the cells were harvested as follows. Before cell cycle analysis, cells were washed with 500 µl PBS and lifted off the well with 300 µl Trypsin-EDTA. Each well was treated separately and all washes and the trypsinized cells for each different condition were pooled in one tube and centrifuged at 300 x g for 5 minutes. The supernatant was aspirated and the resulting cell pellet resuspended in 1 ml hypotonic fluorochrome solution (50 mg/ml propidium iodide in 0.1 % sodium citrate and 0.1 % Triton X-100). Samples were incubated in the dark at 4 °C until flow cytometric analysis (Nicoletti *et al*., 1991), which was carried out within 24 hours.

2.6.2 Immunofluorescence staining of cells for flow cytometry

2.6.2.1 Indirect Immunofluorescence staining (flow cytometry)

2 x10^5 cells were washed and incubated with 100µl of primary antibody for 30mins on ice. Cell surface CD14 expression was detected with undiluted 63D3 or the IgG1 isotype control MOPC 21. Following incubation cells were washed twice before incubated with 100µl secondary-conjugated antibody (goat anti-mouse PE (1:50) or goat anti-rabbit FITC (1:50) for at least 30mins on ice. Cells were either analysed straight afterwards on the flow cytometer.

2.6.2.2 Direct Immunofluorescence staining (flow cytometry)

Cell surface TLR4 was determined using direct immunofluorescence staining with TLR4 (HTA 125) PE or IgG2a/K-isotype control. 2 x 10^5 cells were washed and incubated with 2 µL of HTA 125 or the isotype control for 30mins on ice. Following incubation cells are washed twice and analysed straight away using flow cytometry.

2.7 Immunocytochemical characterisation

Immunocytochemistry is a widely used method for identifying proteins, receptors or other macromolecules in cells or tissue samples by using a specific primary antibody against the immunogen of interest. If the primary antibody is not already conjugated to dye, a secondary dye-labelled antibody (e.g. fluorescent) was used in a second staining step to visualize the expression and/or distribution of the immunogen of interest.

2.7.1 Determination of optimal antibody titer

The definition of an optimum antibody titer is the highest dilution of antibody that gives maximum specific staining with the least amount of background under specific experimental conditions. Often there will be advice from the manufacturer about dilutions, incubation times and temperatures. The quality of staining will be directly influenced by the antibody dilution because if in the rare case of having a too high concentration of primary antibody, the reaction with the antigen is poor due to steric hindrance. When the primary antibody concentration is too low, there is not enough antibody to react with all the antigens presented (Dako, 2006).

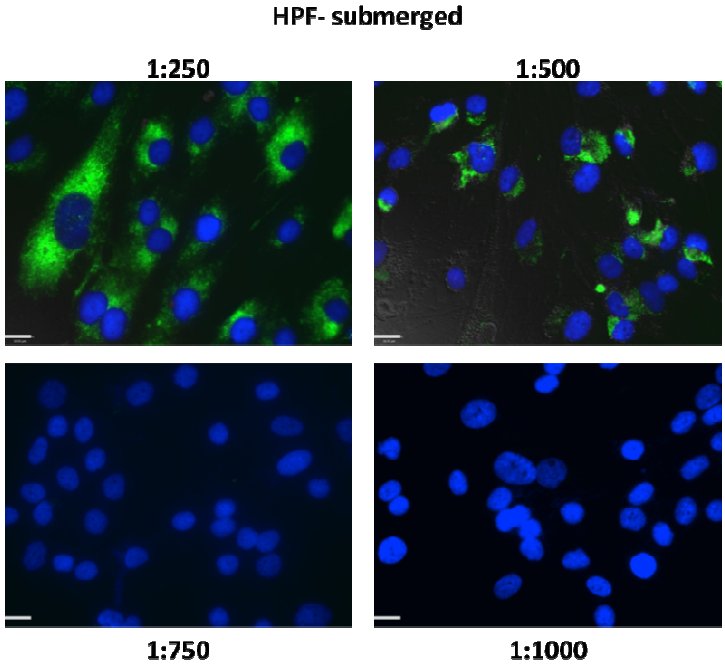

Figure 2.1 Optimising 1B10, an antibody against a fibroblast surface antigen
HPF cells were seeded on human placental collagen type IV coated (10 µg/cm^2) 4 well slides at a cell density of 3.5 x 10^4 cells/ml in 200 µl medium, incubated for 2 days and after fixing and blocking, 1B10 was applied as primary antibody in the following concentrations: 1:250, 1:500, 1:750 and 1:1000 before the secondary goat anti-mouse FITC conjugated antibody was applied. For this antibody the optimum primary antibody dilution is 1:250.

The protocol that has previously been established in our lab for staining epithelial cells for the epithelial cell markers, the cytokeratins (CK), was also adapted and followed for this titration of 1B10 and is described in detail in 2.7.2. Dilutions of antibodies are in general expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. 1:10 means that we put one part of stock solution of antibody into nine parts of diluent. For 1B10 the optimal dilution determined is 1:250, as this gives a minimal background staining with a maximal signal-to-noise-ratio, whereas for 1:500 the antibody was diluted too high, meaning insufficient primary antibody is present to react with all antigen presented in the sample. With any higher dilutions no staining of immunogen is achieved at all.

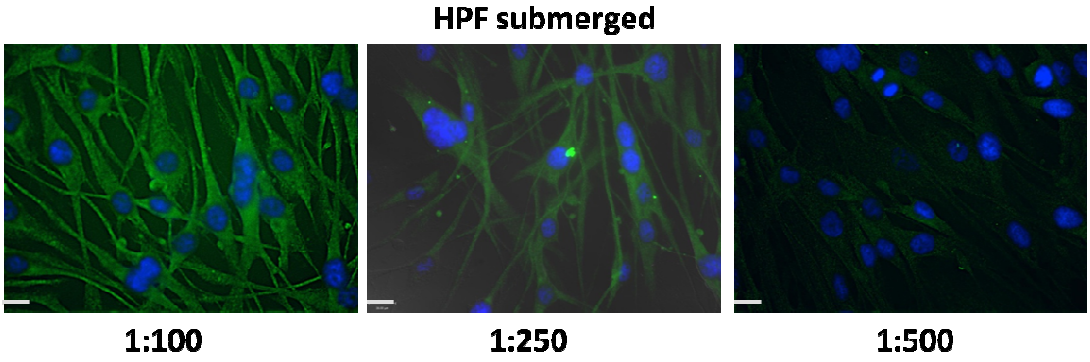

Figure 2.2 Optimising the antibody titer of Vimentin for HPF on 4-well slides
HPF cells were seeded on human placental collagen type IV coated (10 µg/cm^2) 4 well slides at a cell density of 3.5 x 10^4 cells/ml in 200 µl medium, incubated for 2 days and after after fixing and blocking, Vimentin was applied as primary antibody in the following concentrations: 1:100, 1:250 and 1:500 before the secondary goat anti-mouse FITC conjugated antibody was applied. For this antibody the optimum dilution is 1:100 for optimal staining results.

For the anti-vimentin antibody the same method as for 1B10 was performed and the antibody titer to be used was determined at 1:100 to get sufficient staining of the immunogen.

2.7.2 Immunocytochemical characterisation of submerged cell culture on 4 well-slides

Calu-3, IB3-1, C38 and HPF were seeded onto collagen IV coated (10 µg/cm^2) 4 well slides at a cell density of 3.5 x 10^4 cells/ml in 200 µl medium. Cells were incubated at 37 $^{\circ}$C with 5 % CO_2 for 2 days and unless stated otherwise, fixed with 110 µl of 100 % pre-cooled methanol at -20 $^{\circ}$C for 20 minutes. Fixed cells were rinsed three times with 110 µl PBS/ well prior to permeabilising with 110 µl of 0.1 % (v/v) Triton X-100 in PBS for 30 minutes at room temperature. Cells were

rinsed with PBS as before and blocked with 110 µl of 1 % (v/v) normal sheep serum in PBS containing 1 % (w/v) BSA for 1 h at room temperature. After a further 3 washes with PBS, cells were incubated with primary antibodies. All antibodies used were mouse monoclonal antibodies and were directed against cytokeratin 5 (CK5), cytokeratin 8 (CK8), a fibroblast surface protein (1B10), Zonulae occludens 1 (ZO-1) or were isotype-matched control antibody in the form of IgG1, which was used as negative control. The antibodies were diluted to optimal working concentrations in 1 % (w/v) BSA in PBS and again 110 µl were applied to each well. The slides were then incubated overnight at 4 °C. Cells were washed 3 times with PBS, prior to incubation with goat anti-mouse FITC conjugated secondary antibody (1:100), which was applied in 1% BSA in PBS for 1 h at 4 °C in the same total volume as for the primary antibody. Cells were then washed three times with PBS and left to air-dry in the dark prior to mounting with hard set mounting medium containing DAPI. Images were taken on a Zeiss Axiovert 200M fluorescent microscope using objective with 63 x magnification with a DAPI filter (exposures were between 5-20 ms) and a FITC filter (exposures were between 50-200 ms).

2.7.3 Immunocytochemical characterisation of cells cultured on TWs

Cells were grown as described in 2.4.6 and were fixed with 200 µl 100 % pre-cooled methanol at -20 °C for 20 minutes. TWs were washed 3 times with 200 µl PBS before being excised and each membrane was placed, with cells uppermost, in individual wells of a 24 well tissue culture plate. Cells were permeabilised with 100 µl 0.1 % (v/v) Triton X-100 in PBS for 30 minutes at RT and subsequently washed 3 times with 200 µl PBS. Cells were blocked with 100 µl of 1 % (v/v) normal sheep serum in PBS for 1 h at RT. After a further 3 washes with 200 µl PBS, cells were incubated with specific antibodies (as described in 2.7.2). Cells were then washed 3 times with 200 µl PBS before incubation with 100 µl goat anti-mouse FITC conjugated secondary antibody diluted 1:100 in 1 % (w/v) BSA in PBS for 1 h at 4 °C. Cells were further washed with 200 µl PBS and left to air-dry in the dark before mounting with hard set mounting medium containing DAPI. Images were taken on a Zeiss Axiovert 200M fluorescent microscope using objective 63 with a DAPI filter (exposures of 5 - 20 ms) and a FITC filter (exposures of 50-150 ms).

2.8 Histology

The following techniques were performed at Birmingham University in collaboration with Gary Reynolds CSci FIBMS (Institute for Biomedical Research).

2.8.1 Processing of transwells through to paraffin wax

The TWs were formalin fixed by addition of 10% (v/v) formaldehyde to the culture medium at Aston University and directly transferred to Mr Reynolds, who then performed the processing and cutting and staining of sections. Following fixation the TW membranes were carefully cut out of the TWs and bisected. After placing onto biopsy pads and into cassettes, the TW were hand processed through to paraffin wax, comprising of 3 changes in IMS (totalling 2 hours), 3 changes in isopropanol (totalling 3 hours) and impregnation in wax under vacuum (2 hours). The TW were then blocked out and 3µm sections cut, placed onto charged slides and heated for 1 hour at 65°C.

2.8.2 Staining of tissue sections

A standard haematoxylin and eosin stain was performed on sections.

2.8.3 Immunohistochemistry for sections

After dewaxing, rehydration and a peroxidase block, sections underwent a low temperature epitope retrieval technique (ALTER) as previously described (Reynolds *et al*, 2002). Immunostaining was performed on a Dako Autostainer, briefly comprising a 10 minute block in 2% (w/v) casein solution, incubation in optimally diluted primary antibody for 1 hour, Vector ImmPRESS universal secondary for 30 minutes (MP-7500, Vector, UK) and then visualisation with ImmPACT NovaRED chromagen (SK-4805, Vector, UK) for 10 minutes. Following dehydration and clearing sections were mounted with glass coverslips in DPX mount.

2.9 Transepithelial electrical resistance (TER) measurements

TER is a measure of the resistance to ion flux across the epithelial cell layer, which reflects the degree of confluence and the tightness of the cell barrier (Foster *et al*., 2000). TER is commonly used in airway related research working with cell cultures at ALI as it is convenient, reliable and non-destructive to the cell layer. It allows monitoring of the growth of epithelial tissue cultures *in vitro* and to monitor the formation of epithelial cell layer integrity, which also indicates the presence of TJs.

TER of mono- and co-cultures on TWs was measured using an Epithelial Voltohmeter with STX2 chopstick electrodes (World Precision Instruments) to monitor confluence and barrier formation of the epithelial cell layer. As the resistance must be measured between a liquid to liquid interface, 300 µl of medium was added to the apical side of the TW before introducing the

shorter chopstick electrode into the apical compartment and the second and longer electrode into the basal compartment. During initial cell culture of each cell type on TWs the TER was measured on day 0, which is the day ALI was established (apart from HPF, which were always cultured under submerged conditions), day 4, day 7, day 11 and day 14 to generate a TER development profile. This was repeated for each cell type three times. Three individual companion plates, each holding 8 TWs were monitored over 14 days at ALI and each reading was carried out in triplicate. The TER profiles generated were found to be reproducible. Afterwards TER was only measured on day 14 or just before an experiment in order to determine epithelial cell confluence, an intact epithelial cell layer and TJ formation.

TER values reported in this work take into account the area of the cell layer (area of TWs = 0.33cm^2) and are expressed as Ω x cm^2. Triplicate measurements were taken for TW and the background resistance, which was typically between 100-120 Ω x cm^2 (cell-free collagen IV coated TW) was subtracted from the average of a triplicate measurement.

Figure 2.3 Picture of the voltohmmeter and chopstick electrode that was used throughout this project in order to measure TER as an indicator of an intact, functional cell barrier.

2.10 Dot Blot analysis of Mucin secretion - MUC5AC

Apical air surface liquid (ASL) secretions of mono- and co-cultures, as well as basolateral medium, were collected on day 0, 10 and 14 to be analysed for the presence of mucin. The apical sides of mono- and co-cultures were washed with 200 µl sterile PBS (1X), which was transferred from one to the next Transwell® insert (8TWs) to concentrate the mucin present and increase the chances of detection.

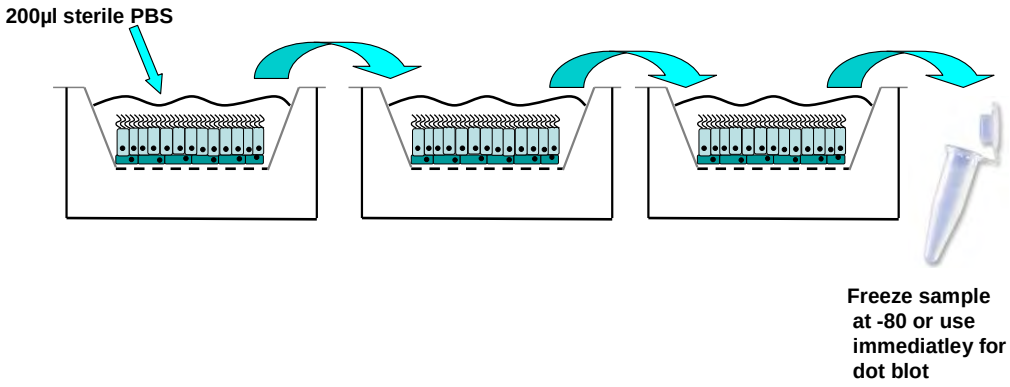

Figure 2.4 Preparation of dot blot samples: wash apical compartment with 200µl sterile PBS and pass this volume from one to the next Transwell® insert to concentrate mucin. Usually eight TWs were used to collect one sample.

The samples were collected, spun down and frozen at -80 °C till analysis. Preparation of the dot blotter included cutting a nitrocellulose membrane to target size and soaking it in distilled water before it was fitted into the dot blotter. A vacuum pump was connected and switched on once all samples had been applied. HPF, C38 and IB3-1 samples were applied in neat and also in a 1:2 dilution, whereas Calu-3 samples were applied in neat, in 1:2 dilution, in 1:5 dilution as well as in a 1:10 dilution to the target wells on the dot blotter with day 10 always leading and day 14 following. In addition to the samples, negative controls were apllied to the blot as well in the following order: PBS, 1 % (w/v) BSA, and the culture medium AEM and DMEM/F12.

After sample application, the membrane was blocked in Tris-buffered saline containing 0.1 % (v/v) Tween-20 (TTBS) with 3 % (w/v) BSA for 1h at RT. TTBS was then used to wash the membrane three times for 15 min prior to overnight incubation at RT on a shaker with primary mouse monoclonal anti-muc5AC antibody at 1:500. The following day the membrane was washed three times for 20 min with TTBS. The secondary antibody used was a horseradish peroxidise linked sheep anti-mouse antibody 1:5000 for 90min at RT on the shaker. Excess secondary antibody was washed off with TTBS (4 x 20 min) before the membrane was treated with enhanced chemiluminescence (ECL) for 5 min to make the visualization of MUC5AC on the membrane possible by detection of the exposed light on a photographic film.

2.11 Zymography

Gelatin zymography is a common method for analysing the expression of matrix metalloproteinase-2 (MMP-2) in cells and media samples. After collecting the samples (carried out in the same manner as for the dot blot) these were either frozen at -80°C or directly prepared for zymography. The 10% gelatin zymograms were bought from Invitrogen (Novex®) and were used as recommended by the manufacturer.

After the gels were mounted in the running chamber and tris-glycine SDS running buffer was added the samples were loaded in non-reducing sample buffer (Novex®) as well as recombinant MMP-2 standard (Peprotech; 62kDa).

After sufficient running time the gel was washed with 2.5% Triton X-100 to remove SDS and renature the MMP-2 species in the gels (4 x 15 min). After preparing the incubation buffer (50mM Tris-HCL, 10mM $CaCl_2$, 1mM $ZnCl_2$, pH 7.6), 2.5% Triton X-100 was poured off and the gel was placed into incubation buffer for at least 72 hours at 37°C. This induced gelatin lysis by renatured MMP-2. On day two, after the incubation buffer has been removed, the gel was incubated in staining solution (1 % (w/v) Coomassie Blue G in, 40 % (v/v) methanol and 10 % (v/v) acetic acid) for 20 minutes, before de-staining in 40 % (v/v) methanol and 10 % (v/v) acetic acid until the gelatinolytic activity was apparent as clear bands on a blue background.

2.12 Electron microscopy

2.12.1 Scanning Electron Microscopy (SEM)

SEM was conducted at The University of Birmingham with the help of Paul Stanley (Manager of the centre for electron microscopy). TWs with either mono- or co-cultures were grown for two weeks at ALI before being fixed with glutaraldehyde (2.5%) for at least one hour. After fixation, the TWs were taken to Birmingham University for further processing. An optional step involving chemical fixation with osmium tetroxide (1 %, 1 h) followed after which the same dehydration steps as for TEM were followed but applying dried ethanol at the last step for dehydration.

The next step, termed critical point drying, involved soaking the specimen in liquid CO_2 for 1 hour and passing through the critical point which is 1170psi/31.1 ^{o}C. Afterwards the samples were mounted on stubs using rapid araldite or double-sided adhesive tape before they are coated with 10 nm gold. Samples were then examined using a SEM and micrographs taken.

2.12.2 Transmission electron microscopy (TEM)

TEM was conducted at The University of Birmingham with the help of Paul Stanley. Transwell® inserts with either mono- or co-cultures were grown for two weeks at ALI before being fixed with glutaraldehyde (2.5 %) for between one hour and several days. During this period, the Transwell® inserts were taken to Birmingham University for further processing. The second step of the chemical fixation involved osmium tetroxide (1 %) for one hour. The fixation process was followed by several dehydration steps using 50 %, 70 %, 90 %, and 100 % alcohol for 2 x 15 minutes at each concentration. Following this 100 % dried alcohol was used for 2 x 15 minutes followed by 2 x 15 minutes with propylene oxide. The embedding process was started on a rotar in a fume cupboard using a mix of propylene oxide/resin (1:1) for 45 minutes and then resin for 1 hour. The TWs membranes were placed just under the surface of fresh resin in embedding moulds. Samples are exposed to a vacuum for 30 minutes before allowing these to return to normal atmospheric pressure. If necessary the samples were orientated in the right way before the resin was polymerised at 60°C for ~ 24 hours. Following this period the sample blocks were trimmed and cut with a glass blade in a microtome to ~1µm sections for light microscope examination. Areas for ultra thin sections were selected and electron microscope grids were prepared (Formvar film/carbon coat). Using a diamond knife 50 -150 nm sections were cut, collected on the grids and stained with uranyl acetate and lead citrate. Examination with TEM followed and micrographs were taken.

2.13 Phenol extraction of LPS

LPS was isolated from *Pseudomonas aeruginosa* 50DR (*P. aeruginosa*) and *Burkholderia cenocepacia* J2315 (*B. cepacia*), which are both CF relevant bacterial strains. These bacteria were obtained from the microbiological culture collection in Aston University's microbiology laboratory. *P. aeruginosa* 50DR was cultured overnight on Mueller-Hinton agar (MHA, Oxoid Ltd) plates at 37 °C. The next day, one colony was picked to be used to inoculate another overnight culture (100ml) in sterile Mueller-Hinton broth (MHB, Oxoid Ltd) at 37 °C at 150 rpm on a shaker. The same procedure was followed for *B. cenocepacia* J2315 except that this strain has to be grown for about 36 h at each step taken. To make sure that the bacterial cultures were pure and clean of contamination, samples were streaked out on a MHA plate. After checking for purity, sterile MHB (50ml) was inoculated and then transferred into 2 conical flasks containing 2 litres of sterile MHB. These were then incubated on a shaker at 37 °C and 150 rpm for 48 h. Another test for purity of the bacterial cultures was carried out (as described above) before each culture was

centrifuged to pellet the bacteria (7871 x g) for 10 min. (Avante JE centrifuge containing using a JA14 rotor). Bacterial pellets were washed with sterile water, centrifuged again and the pellets were re-suspended in 100 ml sterile water. 100 ml of freshly-prepared aqueous phenol (80 %, w/v) at 80 °C was added and the suspensions were stirred at 80 °C for 30 min followed by centrifugation 12298 x g for 20 minutes, using the same centrifuge and rotor as above. Centrifugation yielded 2 distinct phases, the upper phase (containing the LPS) was recovered and transferred into a cellulose acetate dialysis membrane tubing (Sigma), which was sealed and dialysed against running water for 4 days to remove the phenol. After collection of the dialysate, magnesium sulphate was added to a final concentration of 10 mM to encourage LPS micelle formation. After ultracentrifugation (109,564 x g in a Beckmann-Coulter ultracentrifuge for 4 h at 4°C, 70Ti-rotor) the deposited pellet of LPS was re-suspended in 10 ml sterile H_2O and frozen in liquid nitrogen. The LPS was freeze dried, weighed and a stock solution prepared, sterilised by filtration, this was further diluted in sterile AEM (serum free) to make a stock solution of 1 mg/ml which was stored at -20 °C until required.

2.14 Bacterial growth conditions

P. aeruginosa 50DR*, B. cenocepacia* J2315 and *S. aureus* ATCC 6538 were grown in MHB at 37°C and in a shaker at 150rpm or on MHA plates in a 37°C incubator. Bacteria were also stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, UK) at -70 °C until required.

Bacteria were either grown to an optical density of 1, which equals a bacterial density of approximately 1 x 10^9 CFU/ml at 600nm or the bacterial suspension was diluted down to it.

2.15 Antibiotic susceptibility test

In order to determine the susceptibility of *P. aeruginosa 50DR, B. cenocepacia J2315* and *S. aureus* (ATCC 6538) to the antibiotic gentamicin and normocin a disc diffusion test was used. Small filter paper discs containing gentamicin (200 µg/ml) were placed onto MHA plates, which were covered with bacterial suspension using a swab to generate a bacterial lawn. If the bacteria applied were sensitive to gentamicin, a clear zone of growth inhibition was seen around the antibiotic containing filter. The same experiment was carried out with Normocin, which was routinely used in cell culture media to make sure this antibiotic will not inhibit bacterial growth in its working concentration.

2.15.1 Gentamicin susceptibility

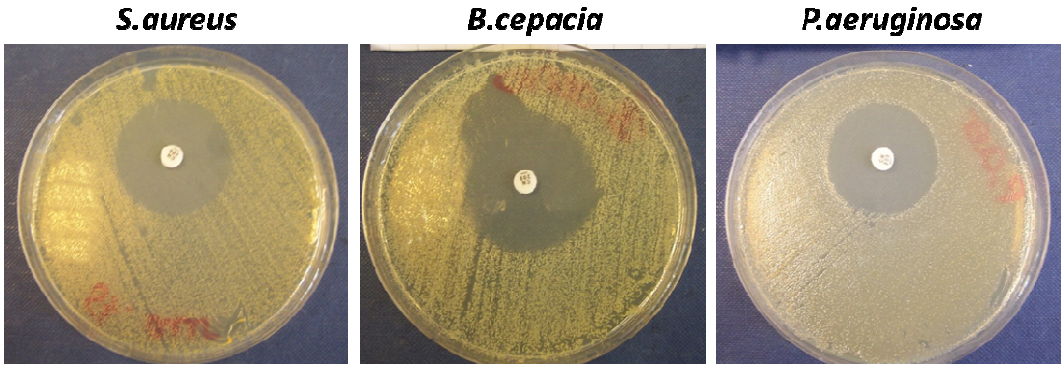

Figure 2.5 Disc diffusion test of gentamicin susceptibility for *S. aureus, B. cepacia* and *P. aeruginosa*
S. aureus, B. cepacia and *P. aeruginosa* cell suspensions were plated out on MHA plates by using a swab to generate a bacterial lawn of each bacterium. The gentamicin disc was dispensed onto the agar. The discs contained 200 µg/ml gentamicin and MHA plates were incubated over night at 37°C before the susceptibility was assessed. All three bacteria show susceptibility as a clear area around each gentamicin disk was shown, where no bacterial growth was identified.

In figure 2.5 it is shown that bacterial cell growth of *S. aureus, B. cepacia* and *P. aeruginosa* was inhibited and clear zones were formed around the Gentamicin disc, which contained 200 µg/ml of gentamicin. This experiment showed that gentamicin can be employed after challenges with live bacteria to kill the extracellular bacteria in the mono- and co-cultures.

2.15.2 Normocin susceptibility

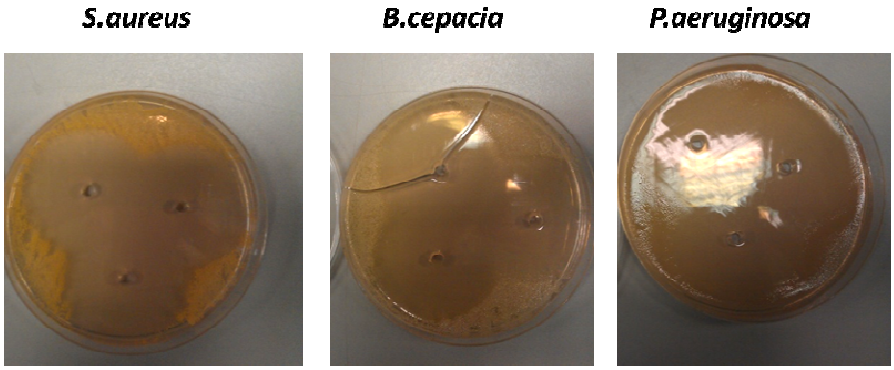

Figure 2.6 Susceptibility of *S. aureus*, *B. cepacia* and *P. aeruginosa* to Normicin at a concentration 50mg/ml
Each bacterium was spread out on a MHA plate using a swab to create a bacterial lawn before holes were cut into the agar using a Pasteur pipette. These holes were then filled with 50µl Normicin. After overnight incubation at 37°C the plates were assessed. For all three bacteria clear areas around the antibiotic filled hole were observed and they are all susceptible to this antibiotic.

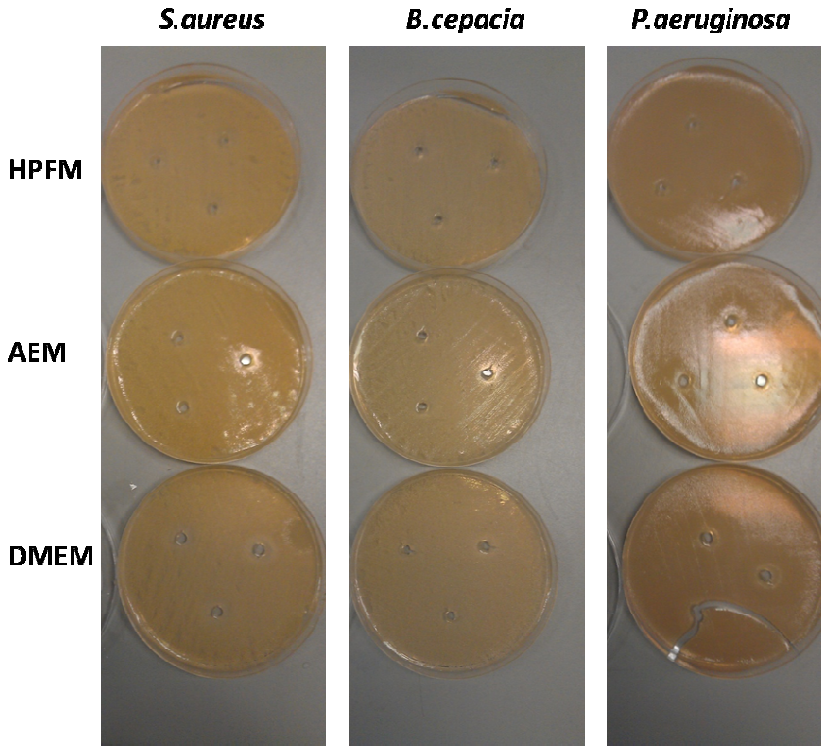

Figure 2.7 Sensitivity of *S. aureus*, *B. cepacia* and *P. aeruginosa* to Normocin at its working concentration of 100µg/ml
At this working concentration none of the bacteria showed clear areas around the holes filled with one of the three media containing Normocin at the working concentration. These bacteria are therefore not susceptible to this antibiotic at this concentration.

In figure 2.6 and 2.7 the inhibitory effects on bacterial growth of *S. aureus, B. cepacia* and *P. aeruginosa* using the antibiotic normocin are shown. In figure 2.6 normocin was employed at 50 mg/ml, which is the stock solution before it is added to cell growth medium. In figure 2.7 it is shown that the antibiotic concentration (100 µg/ml) of normocin at working concentration does not inhibit bacterial cell growth of these three bacteria.

2.16 Heat inactivation of bacteria

Colonies of *P. aeruginosa 50DR, B. cenocepacia J2315* and *S. aureus* were picked from a freshly cultivated MHA plate and grown in 50 ml MHB at 37 °C overnight in a shaker at 150 rpm. On the next day cultures were split into equal parts (one half was kept for experimental treatment of cell cultures with live bacteria) and one half was put into a waterbath maintained at 80 °C for at least 1 h. To be sure bacteria were alive prior to heat-inactivation and dead post heat-inactivation; samples were taken before and after placing the bacterial suspension into the waterbath. Every 15 mins a sample was taken from the waterbath to determine at which time point the bacteria were all killed. After successful heat inactivation the heat-inactivated bacterial cultures were spun down at 3500 x g for 15 min and washed in 10 ml PBS. This was repeated

three times before the culture's optical density (OD) was measured at 470 nm. The OD was then adjusted to an OD_{470nm} of 1.0, which corresponds to a bacterial cell number of approximately 1 x 10^9 cfu/ml for *S. aureus, B. cepacia* and *P. aeruginosa.* These bacterial suspensions were then diluted 1:10 to make a stock solution of 1 x 10^8 cfu/ml. Final bacterial cell density used in the experiments under submerged conditions or at ALI was 1 x 10^7 cfu/ml.

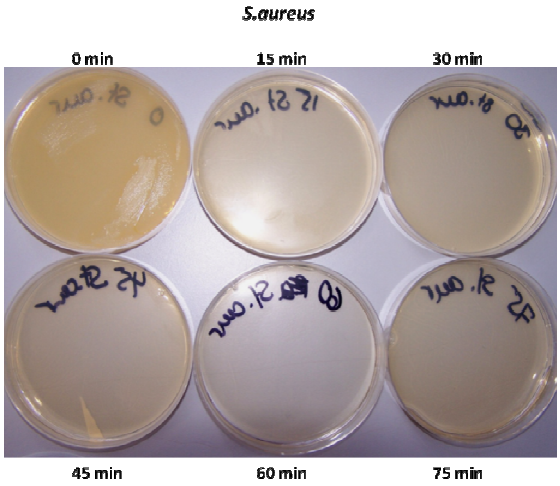

Figure 2.8 Heat inactivation of *S. aureus*
S. aureus was cultured over night in MHB before 20ml of the bacterial suspension was heat inactivated in a waterbath, which was maintained at 80°C for 75 min to evaluate at which time point all bacteria were dead. Samples were taken before heat inactivation and then every 15 min. Samples were plated out on MHA plates and incubated over night at 37°C before these were observed. After 15 min of heat inactivation all bacteria were dead and no colonies were found on agar plates.

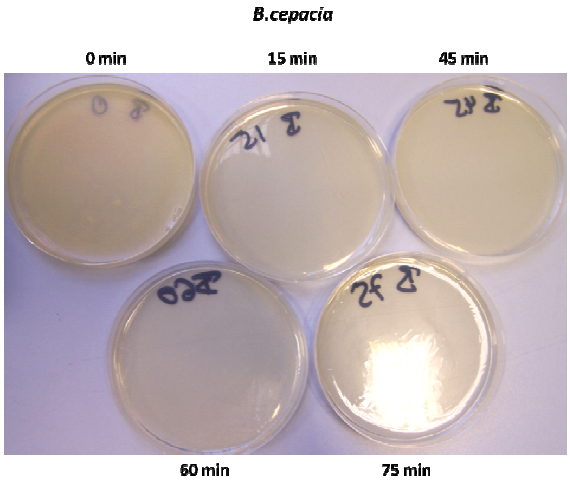

Figure 2.9 Heat inactivation of *B. cepacia*
B. cepacia was cultured over night in MHB. 20ml of the bacterial suspension was heat inactivated in an 80°C hot waterbath for 75 min to evaluate at which time point all bacteria were dead. Samples were taken before heat inactivation and then every 15 min. Samples were plated out on MHA plates and incubated over night at 37°C before these were observed. Agar plates of *B. cepacia* plated before heat inactivation were covered in a thick bacterial lawn, whereas after 15 min of heat inactivation all bacteria were dead and no colonies were found on agar plates.

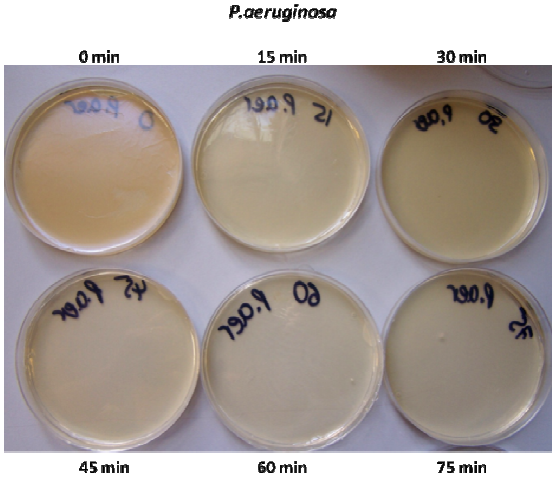

Figure 2.10 Heat inactivation of *P. aeruginosa*
A 50 ml culture of *P. aeruginosa* was incubated over night in MHB and placed on a shaker. 20ml of the bacterial suspension was heat inactivated in an 80°C hot waterbath for 75 min to evaluate at which time point all bacteria were dead. Before heat inactivation one sample was taken and plated out to verify bacteria were alive beforehand. Samples were then taken every 15 min and plated out on MHA plates. After an overnight incubation of the plates at 37°C, plates were visually analysed. before these were observed. Agar plates of *P. aeruginosa* plated before heat inactivation were covered in a thick bacterial lawn, whereas after 15 min of heat inactivation all bacteria were dead and no colonies were found on agar plates.

For all three bacteria, *S. aureus*, *B. cepacia* and *P. aeruginosa* heat inactivation was successful after 15 min at 80°C. For the following experiments employing HIA bacteria, these were in the waterbath for at least 30 min before these were adjusted to the final bacterial density for experiments.

2.17 LPS treatment of submerged mono-cultures and of mono- and co-cultures at ALI

LPS from *P. aeruginosa* 50DR, *B. cenocepacia* and *P. aeruginosa* serotype 10 (obtained from Sigma Aldrich Ltd) were used at 0.1, 1, 10, 100 and 1000ng/ml to treat mono- and co-cultures under submerged or at ALI conditions. The concentration of all stock solutions of LPS types was 1 mg/ml.

For submerged conditions, cells were seeded onto 24-well plates at a cell density of 1 x 10^5 cells/well in full growth medium. After 24 hours incubation under standard conditions the full medium was replaced with serum-free (SF) medium containing ITS supplement (1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin and 0.5 µg/ml sodium selenite; ITS, Sigma), normocin (50mg/ml) and 2mM L-glutamine. For AEM and HPFM, the manufacturer's supplement is added in addition to ITS, as suggested by manufacturer. After another 24 h the

ITS-medium is replaced with ITS-medium containing different concentrations of LPS. Four wells were set up for each concentration of LPS and samples were collected 24 h after LPS exposure for analysis. Cell viability was assessed using CTB as described in 2.4 and Interleukin-8 (IL-8) concentration was measured by ELISA (Peprotech EC Ltd).

The same experimental outline was used for mono- and co-cultures grown at ALI but, based on the results of LPS stimulation of submerged cultures, only the highest concentration of LPS (1000ng/ml) was applied.

2.18 Treatment of submerged mono- cultures and mono- and co-cultures grown at ALI with heat inactivated (HIA) or live bacteria

Submerged mono-cultures were set up exactly the same way as described in 2.17. On the third day ITS-medium was replaced with ITS-medium containing 1 x 10^7 bacteria/ml, either live or HIA (produced as described in section 2.16) and samples were incubated for 24 h. After this incubation all apical supernatants as well as basal media samples were collected for analysis of IL-8 concentrations using an ELISA kit (2.19). For both experimental set ups the cell viability assay CTB was performed as well but with the difference that in the case of treatment with live bacteria an additional step to this experiment was added. The cultures were incubated with 500 µl ITS-medium containing 100 µg/ml gentamicin for one hour, in order to kill any bacteria bound to the cell surface, which is known to kill gram-negative bacteria as well as the gram-positive bacterium *S. aureus*. After the incubation with gentamicin the medium was removed and ITS-medium containing CTB was added to assess cell viability. CTB was used as outlined in section 2.5.

2.19 Detection of Interleukin 8 (IL-8) by ELISA

For the quantification of human IL-8 in cell culture supernatants of submerged and ALI mono- and co-cultures treated with either LPS, heat-inactivated bacteria or viable bacteria, a human IL-8 ELISA development kit, was purchased from Peprotech EC Ltd. (London, UK). All reagents were part of the kit unless otherwise stated and were prepared according to manufacturer's product information. After reconstitution of all reagents as advised in manufacturer's protocol purified anti-human IL-8 capture antibody (0.5 µg/ml) diluted in carbonate bicarbonate coating buffer was applied (100 µl/well) onto high affinity binding 96-microtitre plates (Nunc MaxiSorp™) and incubated over night at 4°C. Afterwards microtitre plates were subsequently washed three times

(300 µl/well) wash buffer (PBS, 0.05 % (v/v) Tween®-20). After removing residual wash buffer 300 µl block buffer (PBS, 1 % (w/v) BSA) was applied to each well and incubated for 1h at RT followed by another wash as described above. The standard (0 pg/ml -10000 pg/ml) and samples for analysis were applied at a volume of 100µl/well to appropriate wells in triplicate and were incubated for 2h at RT. After another wash the detection antibody, anti-human IL-8, was applied at 100 µl/well followed by another incubation of 2 h at RT. Excess antibody was then aspirated and the microtitre plates washed again. An incubation of 30 min at RT with avidin-HRP enzyme solution followed before the microtitre plates were washed five times with wash buffer to make sure any excess avidin-HRP will be washed off. SigmaFAST™ OPD solution was prepared to be ready and applied straight onto each well at 100 µl/well for 5 min at RT. 50 µl/well of a 1M Hydrochloric acid was then added to stop the reaction. A microplate reader at 492nm was used to measure the OD/absorbance of each well. The IL-8 content of samples was calculated based on the standard curve.

3 Chapter 3 - Characterisation of suitable growth substrate and cell culture medium

3.1 Introduction

The overall aim of this project was to develop a co–culture model of CF- and non-CF human airways *in vitro.* In this co-culture model different cell types were grown together and therefore some very important aspects, such as cell growth on a promoting substrate and cell nutrition have to be investigated before moving further ahead. The development of the proposed 3D co-culture model (figure 1.4) using HPF and one of the three epithelial cell lines C38, IB3-1 or Calu-3, at the same time, required a suitable substrate and a suitable medium that ensures healthy cell growth, cell adherence and proliferation of both cell types. The basement membrane (BM) is important for cell adhesion and cell migration (Hinenoya *et al.*, 2008) whereas a suitable medium needs to be determined, which supplies all essential nutrients and growth factors for both cell types to ensure healthy proliferation and differentiation (Moghal and Neel, 1998) of the epithelial cell layer without causing cell cycle changes, such as a drive towards apoptosis.

Airway epithelial cells are supported by the BM, also called basal lamina, *in vivo* and thus an *in vitro* model will need to take account of this feature and incorporate some sort of growth substrate, which is a component of the BM into the model to mimic the *in vivo* situation as close as possible. The BM is a specialised form of extracellular matrix (ECM), which is a complex and very important network, which not only determines the structural organisation of a multicellular organism and its different tissues but it also supports cellular and tissue specific functions (adhesion, migration, proliferation and differentiation) (Teti, 1992, Coraux *et al*., 2008). The ECM is made up of fibrous proteins, such as collagens for structural support, of different adhesion molecules, such as fibronectin and laminin, and of a range of different proteoglycans, which form a “gel like” ground substance together with glycosaminoglycans (GAG) (Teti, 1992). The exact composition of the ECM varies from tissue to tissue. In the respiratory epithelium for example, cells are tightly bound together by tight junctions for example, and only leave little space for ECM to fill, whereas in connective tissues these spaces are large and the ECM abundant. The ECM is not inert, as it was believed, but is constantly being remodelled by cells reshaping and degrading it. There are also proteases (e.g. MMPs) present in the ECM that can change the matrix composition by converting structural molecules to signalling ones by releasing growth factors or other bioactive peptides that have been stored in it (Daley *et al.*, 2008). This turnover is quite high during wound repair, in response to infections and in certain diseases, for

example in cancer (Daley *et al*., 2008, Teti, 1992, Coraux *et al*., 2008). All cells that are in contact with the ECM have cell surface receptors, such as integrins. These will recognize ECM proteins, such as laminin and collagens, and initiate signal transduction pathways, which makes it possible to have a connection between the extracellular space, the cytosol and the cytoskeleton. This way cells can react to changes that happen on the outside of the cell and pass on the information to the inside through the cytosol to the nucleus, where this information than can be used to react to these changes by expression of certain genes for example. This chain reaction leads then to translation and at the end a functional protein that is needed to react to the changes that occurred (Teti, 1992, Daley *et al.*, 2008).

The BM is usually found underlying epithelial cells, including respiratory epithelium, and separating these from the connective tissue underneath (Stanley *et al.*, 1982, Sage, 1982). One of the main components of basement membranes is collagen type IV (Timpl, 1989), which is a network forming type collagen that has a molecular filtration function in contrast to the fibril forming collagens (Kadler *et al.*, 2007). Collagen type IV has been found mainly in the basal lamina of different tissues, including the alveoli in the lungs (Sage, 1982) and has been reported to play an important role in epithelial cell attachment and therefore in maintaining functional integrity (Sage, 1982, Coraux *et al.*, 2008). Another important finding is that human tracheal epithelial cells (HTE) cultured on plastic surfaces lost the ability to produce secretory component (SC), an important transport molecule in the airways but were able to produce it when cultured on collagen IV (Fiedler *et al.*, 1991), which supports the idea of a promoting growth substrate to be used in epithelial cell cultures.

Furthermore it was important to consider the right cell growth medium, which is usually purchased from biotech companies, either in a ready to use version or it can be bought in separates to mix any additives just before use. There is a whole range of different cell growth media available, as different cell types need different nutrients and sometimes special additives to provide a perfect environment for cell proliferation. For different cell types, cell lines and primary cells there are in general recommendations which medium to use. Several researchers have tried in the past to define the exact supplements needed in culture medium to allow epithelial cell differentiation. A list of components has been announced that support viability of freshly isolated cells and serum has been proven useful as it contains TGF-β and has been shown to stop epithelial proliferation and initiate squamous differentiation. Furthermore retinoic acid has been mentioned to appear very important for epithelial cell differentiation as have several others. Depending on the type of epithelial cell type the needs are different as well but one

major factor that seems to work for most cultures to induce differentiation is the establishment of an air-liquid – interface.

As the intention was to co-culture two different cell types, fibroblasts and epithelial cells, together in one cell-culture system, it was vital to determine a suitable cell growth medium, in which both of these cell types can live and proliferate at a normal cell type specific rate without showing significant changes in their cell cycle profile or in their morphology. HPF, C38, IB3-1 and Calu-3 were therefore cultured in three different types of media and their growth characteristics analysed to make sure this important aspect was taken care of.

3.2 Aims

The overall aim of the work in this chapter was to determine a useful substrate and culture medium with which to generate the co-culture model.

In order to achieve this, the first aim was to investigate whether human placental collagen type IV is a useful substrate to provide a basement membrane for cell anchorage and migration that can be used on all tissue culture devices when looking at HPF, C38, IB3-1 and Calu-3 cells.

The second aim was to identify the most suitable culture medium that would support growth and vitality of both cell types without having a negative impact on one of the applied cell types in terms of proliferation, morphology or apoptosis.

3.3 Methods

3.3.1 Cell culture

3.3.1.1 Submerged cell culture

Human pulmonary fibroblasts (HPF) were maintained in fibroblast growth medium 2 containing 11 % FCS (unless otherwise stated), normocin (50mg/ml), 2mM L-glutamine and the manufacturer's supplement (HPFM).

Calu-3 cells were maintained in DMEM/F12, which was supplemented with 15 % FCS (unless otherwise stated), normocin (50 mg/ml) and 2 mM L-glutamine.

IB3-1 and C38 cells were maintained in full medium comprising Promocell® airway epithelial growth medium (AEM) supplemented with 5 % FCS (unless otherwise stated), normocin (50 mg/ml) and the manufacturer's supplement. All cells were cultured in 75 cm^2 tissue culture flasks under standard cell culture conditions at 37 °C and 5 % CO_2 until 90-95 % confluent. Cell morphology, growth, the degree of confluency and confirmation of absence of any bacterial or fungal contamination was assessed using an inverted microscope. The detailed procedure is described in 2.4.4.

3.3.1.2 Cell culture on Transwell inserts

Briefly (detailed in 2.4.6) for mono-cultures of HPF, C38, IB3-1 and Calu-3, cells were seeded apically onto Transwell® Inserts at 3 x10^4 cells/well. Calu-3 cells were seeded in full DMEM/F12, whilst C38 and IB3-1 cells were seeded in AEM. HPF cells were seeded in HPFM. Initially all cells were cultured under submerged conditions with 300 µl medium in the apical compartment and 600 µl medium was added to the basolateral compartment. Four days after seeding cells, the apical medium on the epithelial cells was removed and the apical medium on HPF was refreshed. Basolateral medium was refreshed on the same days for all cells. The basolateral medium and any apical liquid were then collected every 3 - 4 days and fresh medium was added to the basolateral compartment and in case of HPF medium in the apical compartment was also refreshed.

For co-cultures HPF cells were seeded exactly as described above and apical HPFM was removed after 4 days incubation and was replaced with epithelial cell suspension. C38 and IB3-1 cells were seeded on top of the HPF cell layer at the same density (5 x 10^4 cells/well) as Calu-3 but in AEM instead of DMEM/F12. At this point the basolateral medium was changed to the applying epithelial medium used in the co-culture. These cultures were then kept under submerged

conditions for another four days to allow the epithelial cells to form a confluent and adherent layer on top of the HPF. Afterwards the medium was aspirated to establish an ALI and induce differentiation of the epithelial cells.

3.3.2 Cell morphology

Light microscopy was used to monitor cell type specific morphology throughout these experiments.

3.3.3 Cell viability assay- Cell Titer Blue

Cell Titer Blue is a cell viability assay, which is a homogeneous, fluorometric method to monitor cell viability and cell metabolic activity. The blue dye used is termed resazurin (little intrinsic fluorescence activity) and is converted into its highly fluorescent product, called resorufin (pink) in the mitochondria. The fluorescence intensity measured can be correlated directly to the number of viable cells and therefore their metabolically active. Cell Titer Blue was directly applied to the cell medium and required an incubation of 2 h at 37 °C prior to analysis. The fluorescent intensity was measured on a standard multiwell fluorescence spectrophotometer (Spectramax Gemini XS, Molecular Devices, Berkshire UK) at 560 nm excitation, and 590 nm emission wavelength. This method was used to determine an optimum cell density for 96-well plates for a 48 h incubation for each cell line, which was then used for investigating the optimum medium to be used for these co-culture models.

3.3.4 Cell cycle analysis using flow cytometry

Cellular DNA can be stained using propidium iodide (PI), which intercalates with DNA and emits fluorescence and the intensity of the signal correlates with the amount of DNA in each cell.
Cells were seeded in their specific growth medium on 24-well cell culture plates at a density of 1 x 10^5 cells/well and incubated overnight at 37 °C, before their own specific medium was either refreshed, kept (conditioned) or replaced with fresh or conditioned medium of the other cell types. After a further incubation period of 24 h the cells were harvested and the effects of these media to the different cell types was analysed by cell cycle analysis. 4 wells of each different condition were pooled and suspended in hypotonic fluorochrome solution for DNA staining.

Samples were incubated in the dark at 4 °C until flow cytometric analysis (Nicoletti *et al*., 1991), which was carried out within 24 hours (further details in 2.6.1).

3.4 Results

3.4.1 Collagen type IV as a growth substrate

Human placental collagen type IV was used as a growth substrate in this cell culture system. For each cell type, HPF, C38, IB3-1 and Calu-3, the overall quantity of cells attached to the cell culture device was visualised after a 24 h incubation on either collagen IV coated tissue culture devices or plastic surfaces. Light microscopy was used to analyse the different cultures and these images indicate that there were substrate-dependent differences in cell spreading and attachment (figure 3.1).

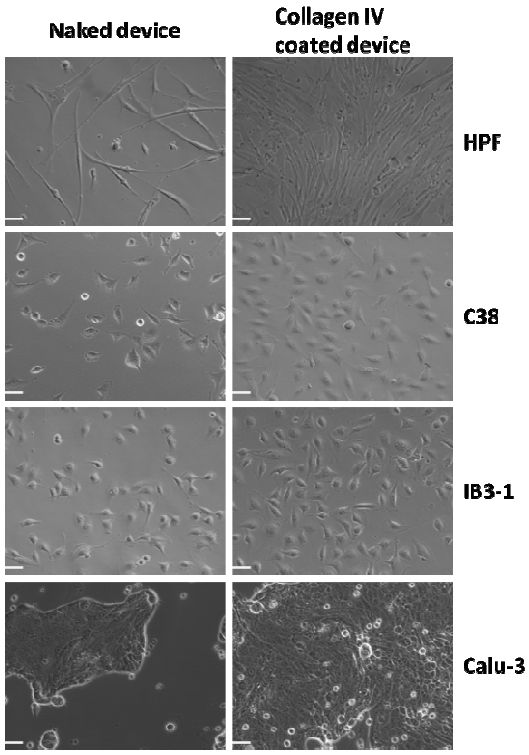

Figure 3.1 Comparison of HPF, C38, IB3-1 and Calu-3 cell growth when cultured on collagen IV coated or uncoated plastics. Cells were seeded on to 24 well plates at a density of $3x10^5$ cells per well and grown for 24hours on naked tissue culture devices or collagen IV coated (10µg/cm^2) plastic. All four different cell types appear to show a much higher confluence after the 24 h incubation on collagen IV, compared to the ones grown on naked devices. Images are representative of three individual experiments (scale bar = 100 µm)

When cells were grown on uncoated plain plastic surfaces, the cells were still sparse after 24 h incubation, whereas, in comparison, for cells grown on collagen IV coated devices, all the different cell types showed a higher number of cells adhered. Figure 3.1 displays the

representative pictures of each cell type under these growth conditions. Pictures on the left side show HPF, C38, IB3-1 and Calu-3 after a 24 h incubation growing on plain plastic surfaces. For all different cell types there are only a small number of cells attached to the plastic surface. Pictures on the right side show cell under the same conditions but grown on Collagen IV and they reveal a clear difference in terms of cell numbers being attached to the collagen IV coated surface. There was definitely a higher number of cells attached for each different cell type.

3.4.2 Investigation of a suitable medium for the co-culture model

The *in vitro* co-culture model that was developed during this project, applied HPF seeded on collagen IV, before one of the epithelial cell lines C38, IB3-1 or Calu-3 were seeded on top. It was essential to investigate in which cell culture medium these two different cell types would grow normally and healthy. If this is the case their cell cycle profile will look normal without showing any dramatic increase in cell proliferation, without showing any morphological changes or equally important without increase in cell death (apoptosis).

3.4.2.1 Cell morphology of HPF, C38, IB3-1 and Calu-3 after exposure to different cell culture medium

All cell types used were seeded onto collagen IV coated 24 well plates using their cell type specific medium for 24 h to allow cell adherence and proliferation. Afterwards the cell type specific medium was removed and was either kept (in order to use as "conditioned medium"), or was refreshed with the same cell-type specific medium, or replaced with one of the other two media (figure 3.2). This meant that each cell type was exposed to fresh and conditioned medium of each kind. 24 h incubation followed before the cells were analyzed for morphological changes and level of confluence using a light microscope.

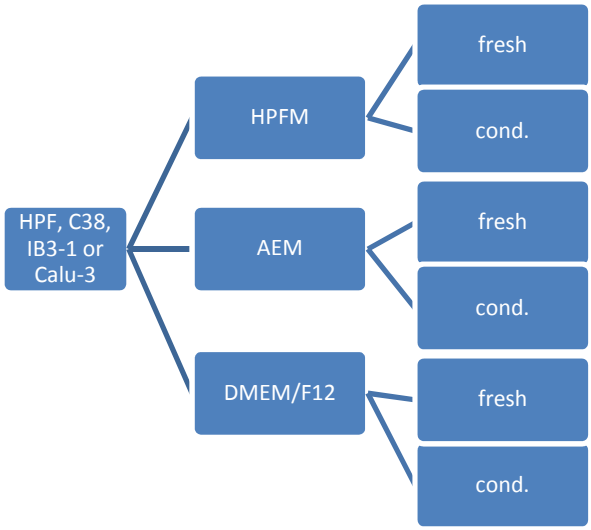

Figure 3.2 Flow diagram of experimental set up for investigation of a suitable medium for the proposed co-culture model The diagram shows that every cell type was exposed to fresh and conditioned human pulmonary fibroblast medium (HPFM), airway epithelial medium (AEM) and to Dulbecco's modified eagle medium: nutrient mixture-12 (DMEM/F12).

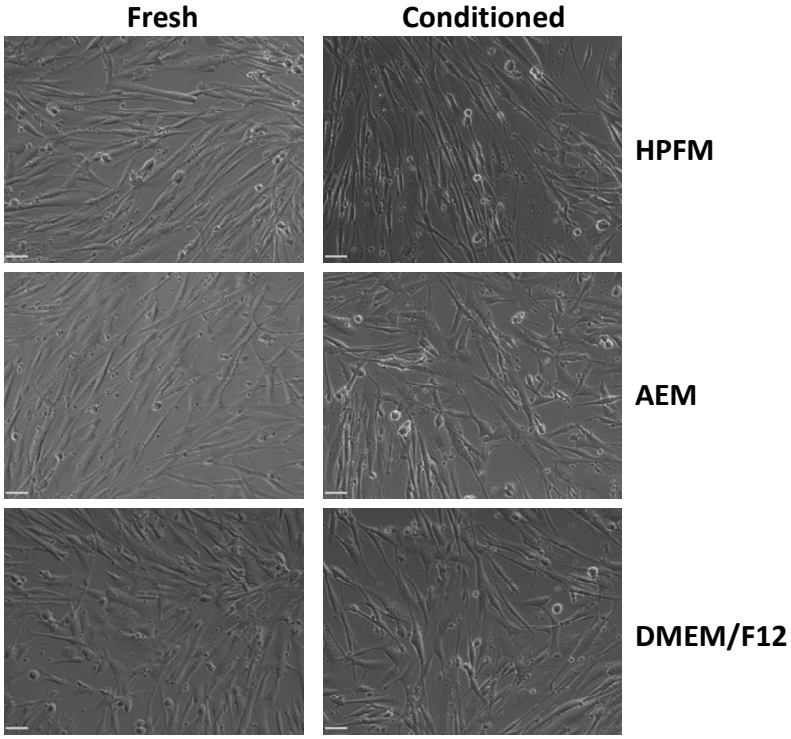

Figure 3.3 HPF cell morphology after exposure to different cell growth medium
HPF were seeded onto collagen IV coated 24 well plates at a density of $1x10^5$ cells/well and incubated for 24 h before the cell specific medium was replaced with either fresh or conditioned HPFM, AEM or DMEM/F12. After further 24 h incubation the pictures were taken. On all pictures HPF cells look morphologically the same and the confluency looks even across the different conditions. Images are representative of three individual experiments (scale bar = 100 µm)

The pictures in figures 3.3 show HPF cell morphology after being cultured in all different media applied. The two pictures in the top row show HPF cultured in HPFM in fresh (left side) and in conditioned form (right side). HPF on both pictures show the same spindle like morphology and the same degree of confluence. In the next two pictures (middle) the morphology and confluency of HPF can be seen after they were cultured in fresh (right side) or conditioned (left side) AEM. The morphology and confluency looks exactly the same as in the pictures above when cultured in HPFM. The same result is shown for culturing HPF in DMEM/F12 again in a fresh (left side) and conditioned (right side) form. After analysing all epithelial cells in all different media applied, as well, it becomes clear that morphology and confluency are not affected by any of these media for any of these cells, when looking at the cell populations using light microscopy after 24 h incubation. Cell morphology of C38, IB3-1 and Calu-3 is shown in figure 3.4- 3.6.

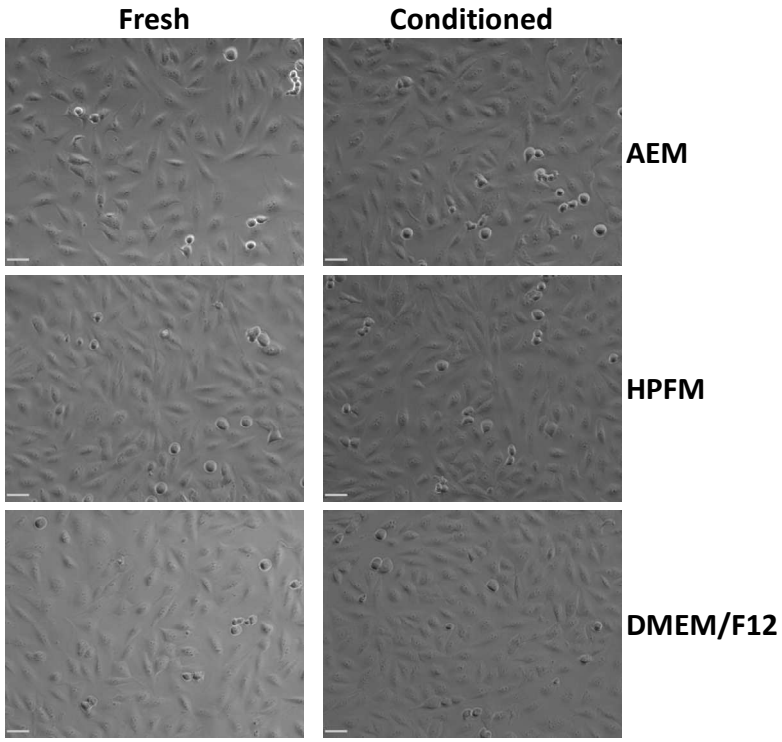

Figure 3.4 C38 cell morphology after exposure to different cell growth medium
C38 were seeded onto collagen IV coated 24 well plates at a density of $1x10^5$ cells/well and incubated for 24 h before the cell specific medium was replaced with either fresh or conditioned AEM, HPFM or DMEM/F12. After further 24 h incubation these epithelial cells look morphologically the same on all six pictures and the confluency looks even across the different conditions. Images are representative of three individual experiments (scale bar = 100 µm)

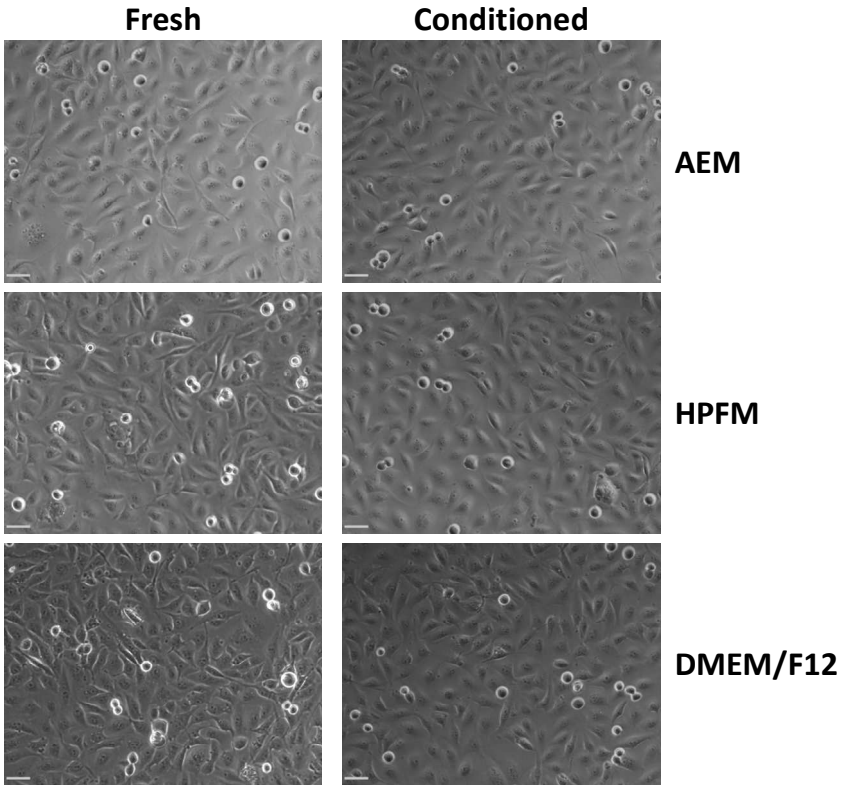

Figure 3.5 IB3-1 cell morphology after exposure to different cell growth medium
After seeding IB3-1 onto collagen IV coated 24 well plates at a density of $1x10^5$ cells/well, the plates were incubated for 24 h before the cell specific medium was replaced with either fresh or conditioned AEM, HPFM or DMEM/F12. After 24 h incubation under these conditions IB3-1 look morphologically the same on all six pictures and showed a healthy looking confluent layer of cells. Images are representative of three individual experiments (scale bar = 100 µm)

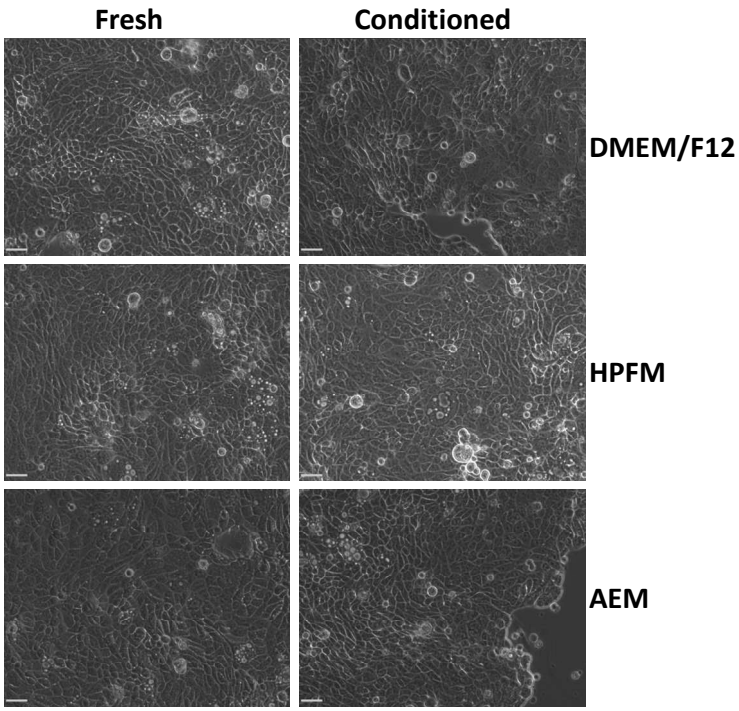

Figure 3.6 Calu-3 morphology after exposure to different cell growth medium
Calu-3 were seeded at a density of $1x10^5$ cells/well onto collagen IV coated 24 well plates and incubated for 24 h before the cell specific medium was replaced with either fresh or conditioned DMEM/F12, HPFM or AEM. After further 24 h incubation the morphology and the degree of confluency was analysed. Under all different conditions the same result could be seen: normal morphology and confluency looks even across the different conditions. Images are representative of three individual experiments (scale bar = 100 µm)

3.4.3 Cell viability assay – Cell Titer Blue® (CTB) assay

3.4.3.1 Optimising the cell viability assay for HPF, C38, IB3-1 and Calu-3

Before setting up 96-well plates for the cell viability experiment, a cell dilution experiment was carried out to find the optimum cell densities to seed in each well for each of these cell types. The cell density had to be taken into account as cells were to be grown for 48h (in total) in a 96-well plate. If there are too many cells there will be a decrease of metabolic activity (low fluorescent signal) due to the lack of nutrients and that could be interpreted with a wrong result. If there are too few cells in each well, e.g. 50 cells and in the next one 100 cells the difference in fluorescent intensity is not high enough to be able to monitor little changes in cell metabolic activity, even though there is a direct correlation of cell number and fluorescent signal, it is not sensitive enough for such small numbers. The optimum cell number needs to be high enough to give a fluorescent signal higher than the background reading but also leave enough range to detect dramatic increases of metabolic activity.

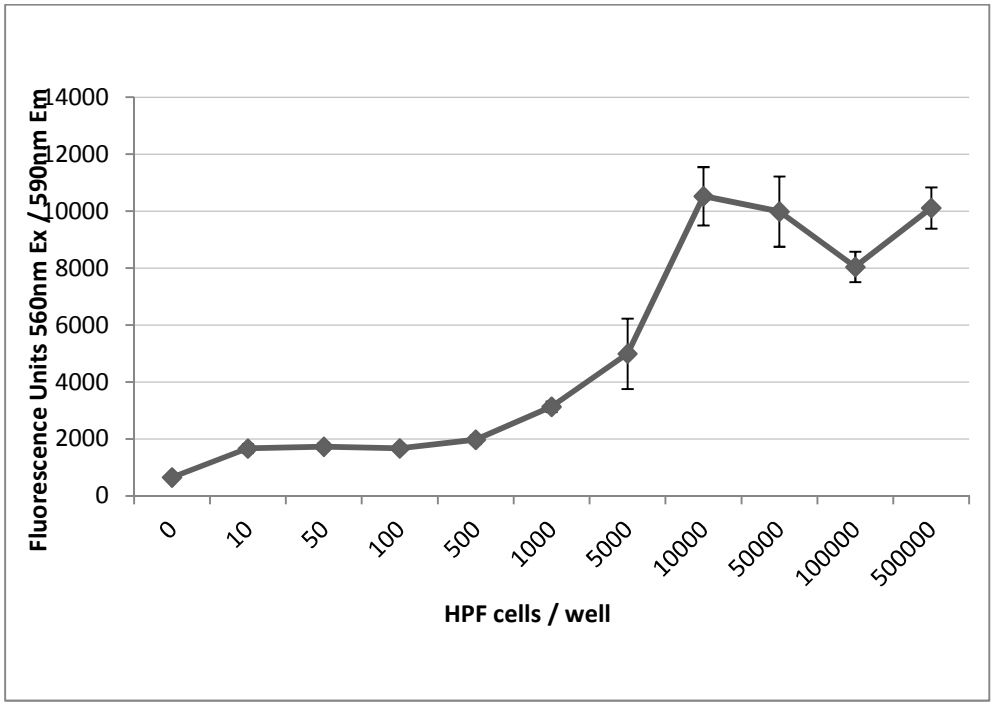

Figure 3.7 Optimising cell densities for optimum cell growth of HPF on 96-well plate
HPF were seeded onto 96-well plates at the indicated density per well (x-axis) and incubated for 48h to find an optimum cell number that can be grown in these wells without showing cell overgrowth and/or apoptosis. After the 48h incubation CTB was added and following manufacturer's guide incubated for 2h before reading the 96-well plate on a fluorometer at 560 nm excitation and 590 nm emission (4 separate wells per cell density were used for analysis). The optimum cell density for these cells was 5 x 10^3 cells/well, which resulted in an average of 4997 ± 1236 FU. Each bar represents the mean ± SD from three different experiments.

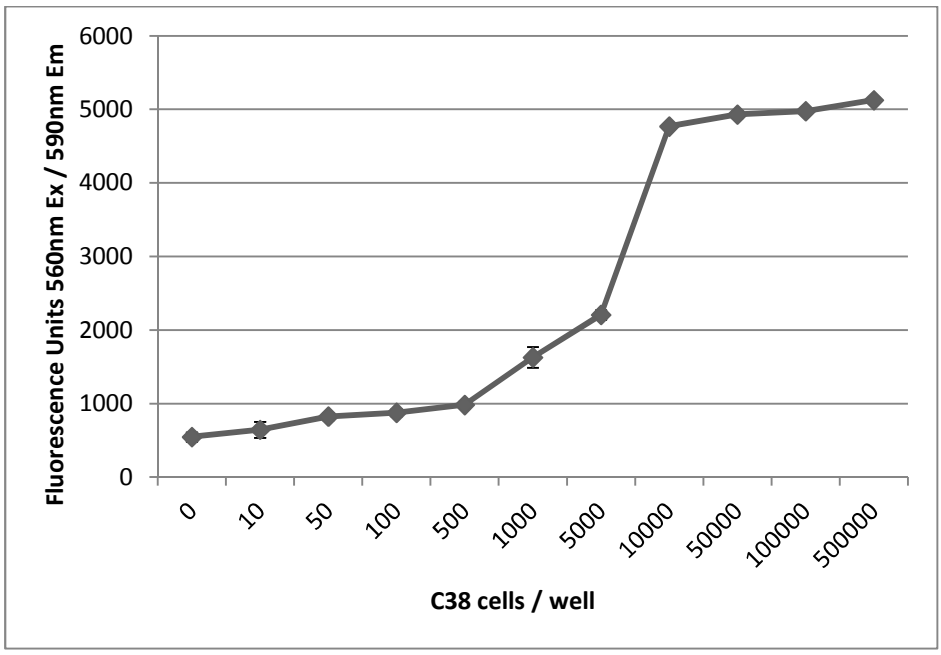

Figure 3.8 Optimising cell densities for optimum cell growth of C38 on 96-well plate
C38 were seeded onto 96-well plates at different cell densities (x-axis) and incubated for 48h to determine an optimum cell number for this experiment without having an effect on cell growth and/or apoptosis. After the 48h incubation CTB was added and following manufacturer's guide incubated for 2h before reading the 96-well plate on a fluorometer at 560 nm excitation and 590 nm emission (4 separate wells per cell density were used for analysis). 5 x 10^3 cells/well is the optimum cell density to be seeded with an average FU of 2208 ± 65 for C38. Each bar represents the mean ± SD from three different experiments.

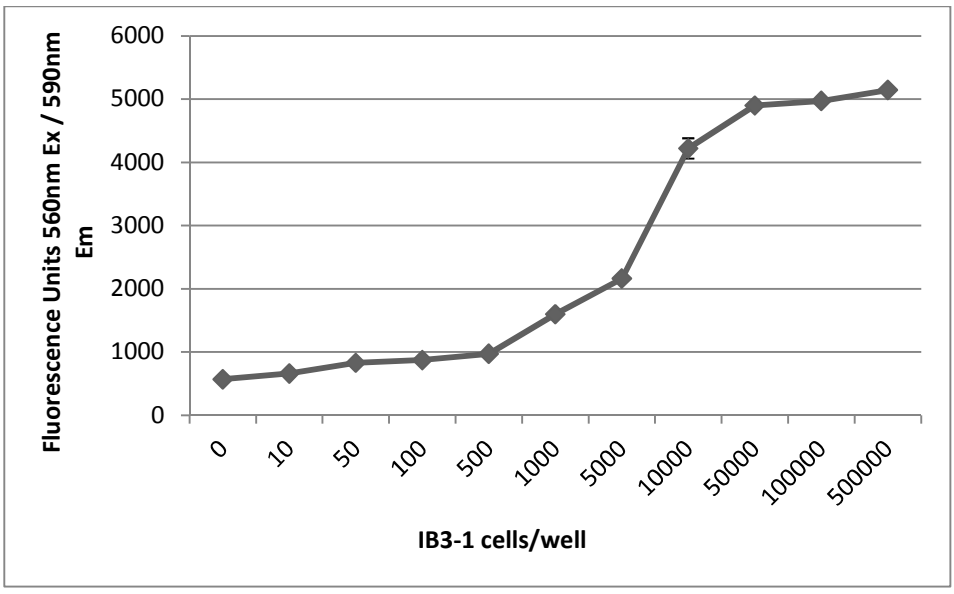

Figure 3.9 Optimising cell densities for optimum cell growth of IB3-1 on 96-well plate
A 96-well plate was used to carry out this experiment and IB3-1 were seeded at different cell densities (x-axis) and incubated for 48h to determine an optimum cell number for this experiment without having an effect on cell growth and/or apoptosis. After the 48h incubation CTB was added and following manufacturer's guide incubated for 2h before reading the 96-well plate on a fluorometer at 560 nm excitation and 590 nm emission (4 separate wells per cell number were used for analysis). The optimum cell density used was 5 x 10^3 cells/well and gave an average FU of 2162 ± 33 for IB3-1. Each bar represents the mean ± SD from three different experiments.

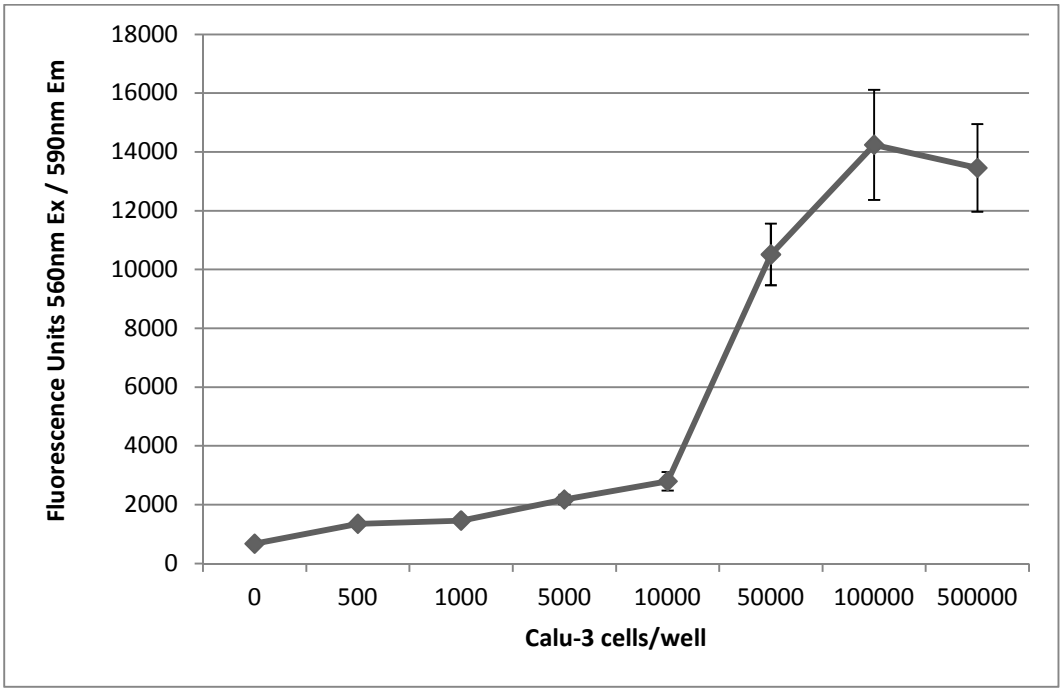

Figure 3.10 Optimising cell densities for optimum cell growth of C38 on 96-well plate
Calu-3 were seeded onto 96-well plates at the indicated cell densities and incubated for 48h to find an optimum density of cells that can be grown on this surface area without showing cell overgrowth and apoptosis. After the 48h incubation CTB was added and following manufacturer's guide incubated for 2h before reading the plate on a fluorometer at 560nm excitation and 590nm emission (4 wells per cell number).The optimum cell density determined was 5 x 10^4 cells/well resulting in an average FU of 10511 ± 1048. Each bar represents the mean ± SD from three different experiments.

For HPF the optimum cell density determined in this experiment was 5 x 10^3 cells/well, which resulted in an average FU of 4997 ± 1236 (figure 3.7). For the epithelial cell lines C38 and IB3-1 the optimum cell density determined was 5 x 10^3 cells/well with an average FU of 2208.225 ± 65.24 for C38 (figure 3.8) and an average FU of 2162 ± 33 for IB3-1 (figure 3.9).The optimum density of cells for Calu-3 was 5x10^4 cells/well resulting in an average FU of 10511 ± 1048 (figure 3.10).

3.4.3.2 Medium investigation using CTB

After determination of the optimum cell densities (figure 3.7 – 3.10) for using 96-well plates for the CTB assay, cells were seeded at the optimum densities and incubated for 24 h each in their own cell type specific medium. After this all cell types were challenged with fresh and conditioned HPFM, AEM, and DMEM/F12, following the same experimental set up as shown in figure 3.2.

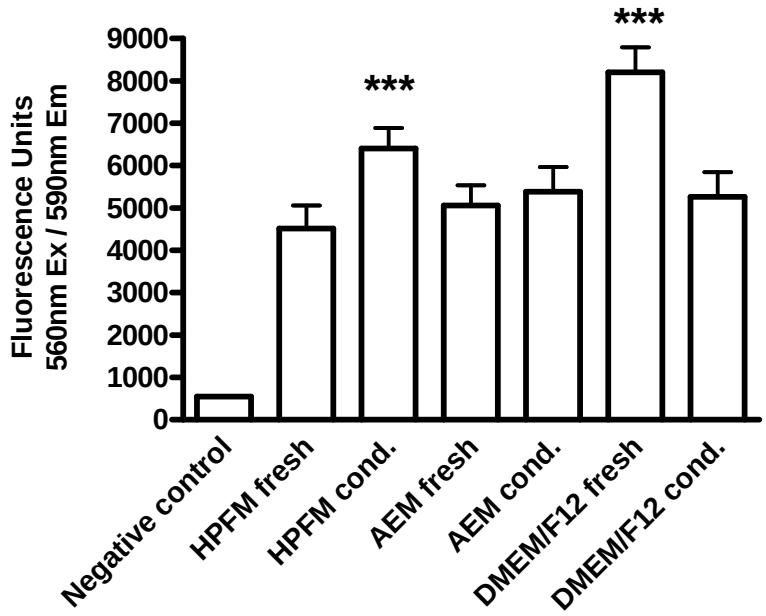

Figure 3.11 Cell viability of HPF cultured in different fresh and conditioned cell growth medium
HPF were seeded onto 96-well plates at a density of $5x10^3$ cell/well and incubated in HPFM for 24h before challenging cells with either fresh or conditioned HPFM, AEM or DMEM/F12. After further 24 h incubation CTB was applied for two hours before the fluorescence was determined using a fluorometer at 560 nm excitation and 590 nm emission respectively. HPF cells show a significant increase of metabolic cell activity in conditioned HPFM and fresh DMEM/F12, when compared to the positive control, HPF in fresh HPFM.
Results have been subjected to 1way –ANOVA and Tukey's test, ***= $p < 0.01$ compared to positive control, where the positive control was HPF grown in their specific fresh HPFM for the duration of the experiment. Negative control was fresh HPFM without any cells present. Data are presented as mean + standard deviation and results are taken from three independent experiments looking at 8 wells in each experiment.

HPF cells were grown in either fresh or conditioned medium of each kind after they were seeded onto 96 well plates and incubated for 24h in their own fresh HPFM before they were challenged with fresh and conditioned AEM or DMEM/F12. The results for HPF show that there is a significant increase from 4522 ± 533 FU in fresh HPFM to 6399 ± 488 FU in conditioned HPFM. When HPF were grown in fresh DMEM/F12 there was also a significant increase to 8205 ± 590 compared to the positive control of HPF cells in HPFM. For fresh and conditioned AEM, as well as conditioned DMEM/F12, no significant change in fluorescent intensity could be determined compared to HPF in fresh HPFM (figure 3.11).

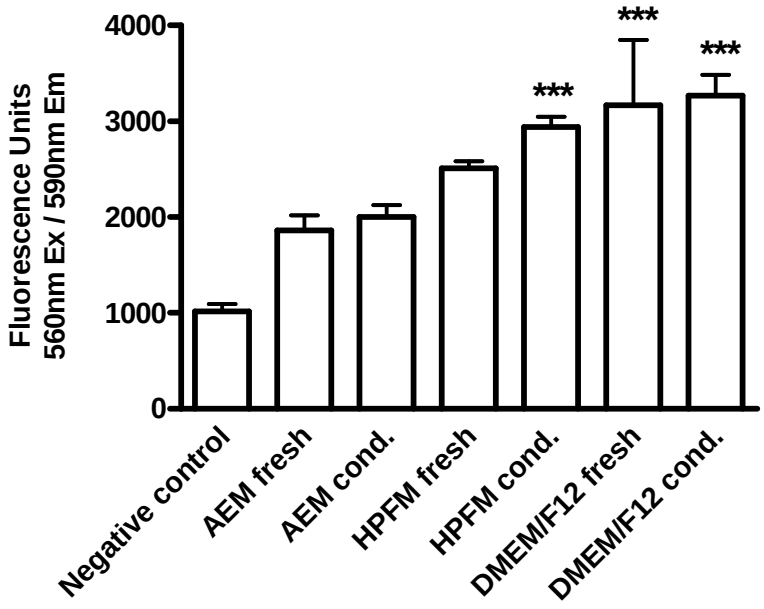

Figure 3.12 Cell viability of C38 cultured in different fresh and conditioned cell growth medium
C38 were seeded onto 96-well plates at a density of $5x10^3$ cell/well and incubated in AEM for 24h before exchanging the medium with either fresh or conditioned AEM, HPFM or DMEM/F12. After a further 24 h incubation CTB was applied for two hours before the fluorescence signal was measured using a fluorometer at 560 nm excitation and 590 nm emission respectively. C38 cells show a significant increase of metabolic cell activity when grown in conditioned HPFM or in fresh or conditioned DMEM/F12.
Results have been subjected to 1way –ANOVA and Tukey's test, ***= $p < 0.01$ significance compared to positive control, where the positive control was C38 grown in their specific fresh AEM. Data presented as mean + standard deviation and results taken from three independent experiments looking at 8 wells in each experiment.

For the epithelial cell line C38 (figure 3.12) no significant differences in the fluorescent intensity were measured for conditioned AEM or fresh HPFM, when compared to the positive control, which was C38 in fresh AEM. A significant increase of the fluorescent signal measured was seen when C38 were grown in conditioned HPFM. Here, the fluorescent signal increased from 1861.25 ± 158.3 FU to 2942.88 ± 104.58 and when C38 were grown in DMEM/F12 a significant increase in FU for fresh (3168.8 ± 679.52 FU) and conditioned medium (3266.99 ± 219.64 FU) was detected.

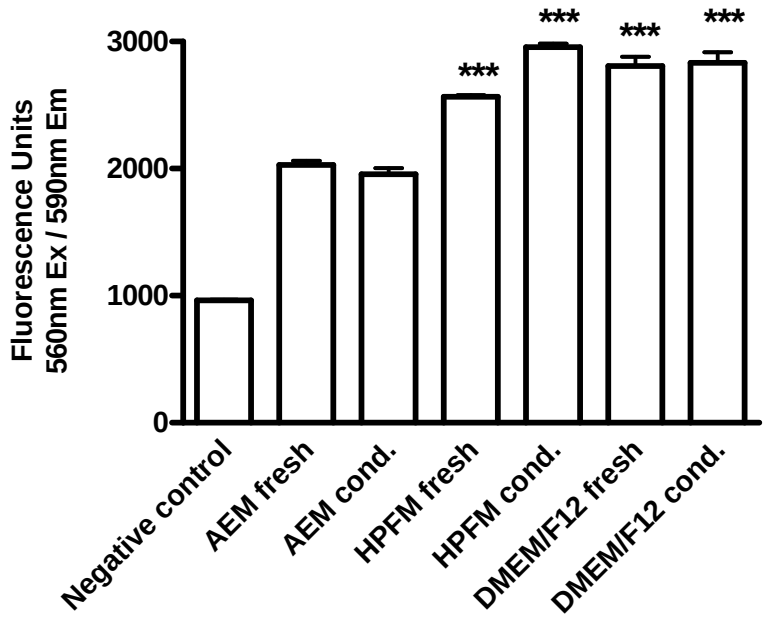

Figure 3.13 Cell viability of IB3-1 cultured in different fresh and conditioned cell growth medium
IB3-1 were seeded onto 96-well plates at a density of $5x10^3$ cells/well and incubated in AEM for 24h before exchanging the medium with either fresh or conditioned AEM, HPFM or DMEM/F12. After a further 24 h incubation CTB was applied for two hours before the fluorescence signal was measured using a fluorometer at 560 nm excitation and 590 nm emission respectively. IB3-1 cells show a significant increase of metabolic cell activity when grown in fresh or conditioned HPFM or when grown in fresh or conditioned DMEM/F12.
Results have been subjected to 1way –ANOVA and Tukey's test, ***= $p < 0.01$ significance compared to positive control, where IB3-1 was grown in their specific fresh AEM. The negative control was AEM without any cells present. Data presented as mean + standard deviation and results taken from three independent experiments looking at 8 wells in each experiment.

The fluorescent signal measured for IB3-1 cultured in fresh AEM is 2029 ± 32 and there is no difference when comparing it to cells grown in conditioned AEM. Significant differences were found for all the other media tested. When grown in fresh HPFM the fluorescent signal increased to 2565 ± 13 and when grown in conditioned HPFM the signal reached 2957 ± 27. A significant difference compared to control was also measured for IB3-1 grown either fresh DMEM/F12 (2808 ± 74 FU) or conditioned DMEM/F12 (2831 ± 84 FU).

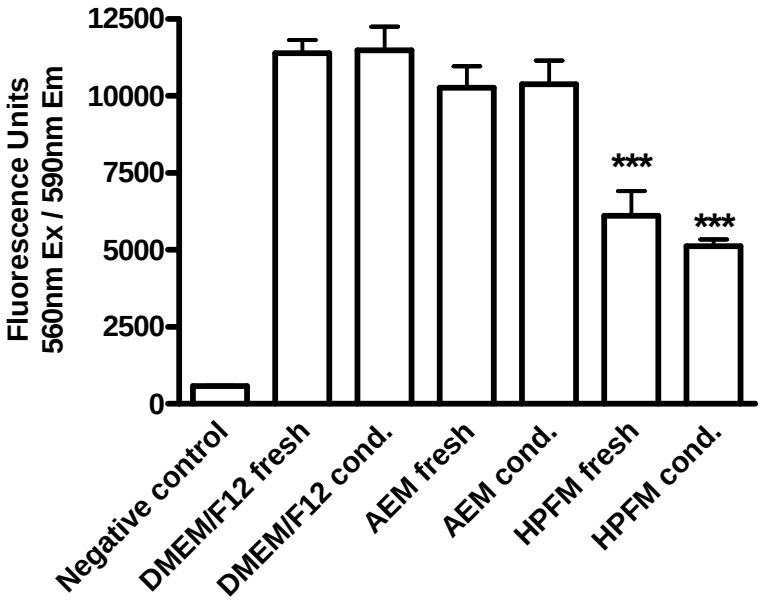

Figure 3.14 Cell viability of Calu-3 cultured in different fresh and conditioned cell growth medium
Calu-3 were seeded onto 96-well plates at a density of $5x10^4$ cell/well and incubated in DMEM/F12 for 24h before exchanging the medium with either fresh or conditioned DMEM/F12, AEM or HPFM. Cells were incubated for another 24h before CTB was applied for two hours. Plates were read on a fluorescent plate reader at 560nm excitation and 590nm emission.
Results have been subjected to 1way –ANOVA and Tukey's test, ***= $p < 0.01$ significance compared to positive control, which is Calu-3 in DMEM/F12. Data presented as mean + standard deviation and results taken from three independent experiments looking at 8 wells in each experiment.

Calu-3 cells were assessed for their ability to grow in the indicated media using CTB as well and the results in figure 3.14 show that there was no significant difference in the fluorescent signal measured for conditioned DMEM/F12 (11483 ± 774 FU), for fresh (10267 ± 697 FU) or conditioned (10385 ± 763 FU) AEM, when compared to fresh DMEM/F12, which resulted in a reading of 11395 ± 424 FU. However, when cultured in fresh or conditioned HPFM a significant decrease of fluorescence was observed, where the signal went down to 6114 ± 797 FU for the fresh medium and down to 5124 ± 212 FU for the conditioned medium.

	Fresh HPF	Cond. HPF	Fresh AEM	Cond. AEM	Fresh DMEM /F12	Cond. DMEM /F12
HPF	control	↑	—	—	↑	—
C38	—	↑	control	—	↑	↑
IB3-1	↑	↑	control	—	↑	↑
Calu-3	↓	↓	—	—	control	—

Table 3.1 Summary of the viability assay results for HPF, C38, IB3-1 and Calu-3 after exposure to fresh and conditioned media for 24 h
The table shows the effects that fresh or condioned media had on the different cell types when compared to the control, which was each cell type in their own specific medium. Significant increase in metabolic activity is indicated by an arrow facing up, whereas decreases of metabolic activity are indicated by a down facing arrow and no changes are shown as hyphen.

Table 3.1 summarises the results of the cell viability assay, which was performed to investigate the effect of different media in either fresh or conditioned form on the cell's metabolic activity after a 24 h incubation in these media. The arrows in the table show when a medium had a significant impact on cell metabolic activity either in an increasing or decreasing fashion. If there was no significant difference of metabolic activity compared to the control it is indicated by a hyphen.

3.4.4 Cell cycle analysis using flow cytometry after exposure to different culture media

3.4.4.1 Cell cycle analysis of HPF

HPF's specific cell cycle profile was generated after cells were cultured in fresh HPFM, to have a baseline cell cycle profile to compare the other profiles with that were generated after HPF were cultured in the other applied media, AEM and DMEM/F12. The experimental set up in terms of which cell type was grown in what medium is shown in figure 3.2.

The distribution of HPF cells in the different cell cycle phases shows the typical cell cycle pattern with almost no cells in subG0 (table 3.2). When using flow cytometry, the labeled cells that run through it and are detected by the fluorescent signal coming off them from the PI-staining of DNA as well as by their size and granularity. These cells are then plotted on a dot plot according to their size and granularity and can be gated in a certain region, which is usually tightly drawn around the main population of cells on that dot plot. The intention of gating the main cell population is to discriminate any debris, doublets and cell aggregates, which are scattered around this main cell population. A histogram is also produced showing each cells fluorescent intensity (proportional to DNA amount in nucleus). The histogram can then be divided into the cell cycle phases and the cell counts plus mean fluorescence for each region will be analysed by the flow cytometer.

Cell cycle phase	Percent of gated cells	Std. Dev.
subG0	1.30%	0.57
G0,G1	57.50%	5.05
S-phase	13.20%	1.65
M,G2	27.50%	4.37

Table 3.2 Normal distribution of HPF cells across the cell cycle when cultured in fresh HPFM

In table 3.1 the exact distribution of HPF cells (in % of gated cells) for this cell cycle profile is given after being cultured in fresh HPFM, whereas in figure 3.15 the cell counts for each cell cycle phase of HPF cells in each of the applied media is presented. Every profile generated by culturing HPF in all the media applied is compared to the original profile, HPF cultured in HPFM, (dark green bars). For none of the applied media, whether in fresh or conditioned form, a significant difference was observed.

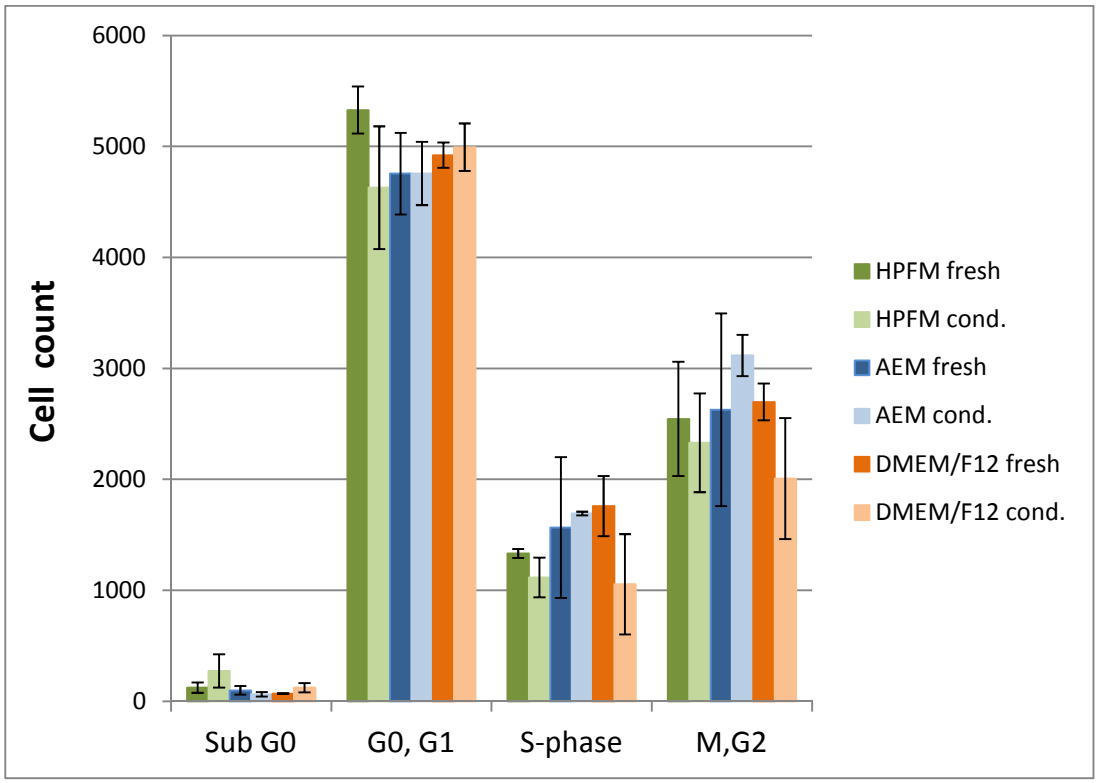

Figure 3.15 Cell cycle analysis of HPF after a 24 h exposure to HPFM, AEM and DMEM/F12 in fresh and conditioned form.
The 24 h exposure of HPF cells to these media does not cause any increase in SubG0 as there is no significant difference in the number of cell present in the particular area defined as Sub G0. No cell cycle shift can be observed in this graph for any of the media. Each bar represents the mean ± SD from three different experiments.

3.4.4.2 Cell cycle analysis of C38

C38 were investigated for their ability to life and grow in HPFM and DMEM/F12 and results were compared to the cell cycle profile produced when grown in AEM as their specific culture medium.

Cell cycle phase	Percent of gated cells	Std. Dev.
subG0	1.10%	0.32
G0,G1	65.20%	0.73
S-phase	13.10%	0.61
M,G2	19.20%	0.46

Table 3.3 Normal distribution of C38 cells across the cell cycle when cultured in fresh AEM

The distribution for cells in the cell cycle profile for fresh AEM is shown in table3.2. Only 1.1% percent of the cells were found in subG0, 65.2% in G0/G1 and about one third in S-phase and M/G2 phase taken together.

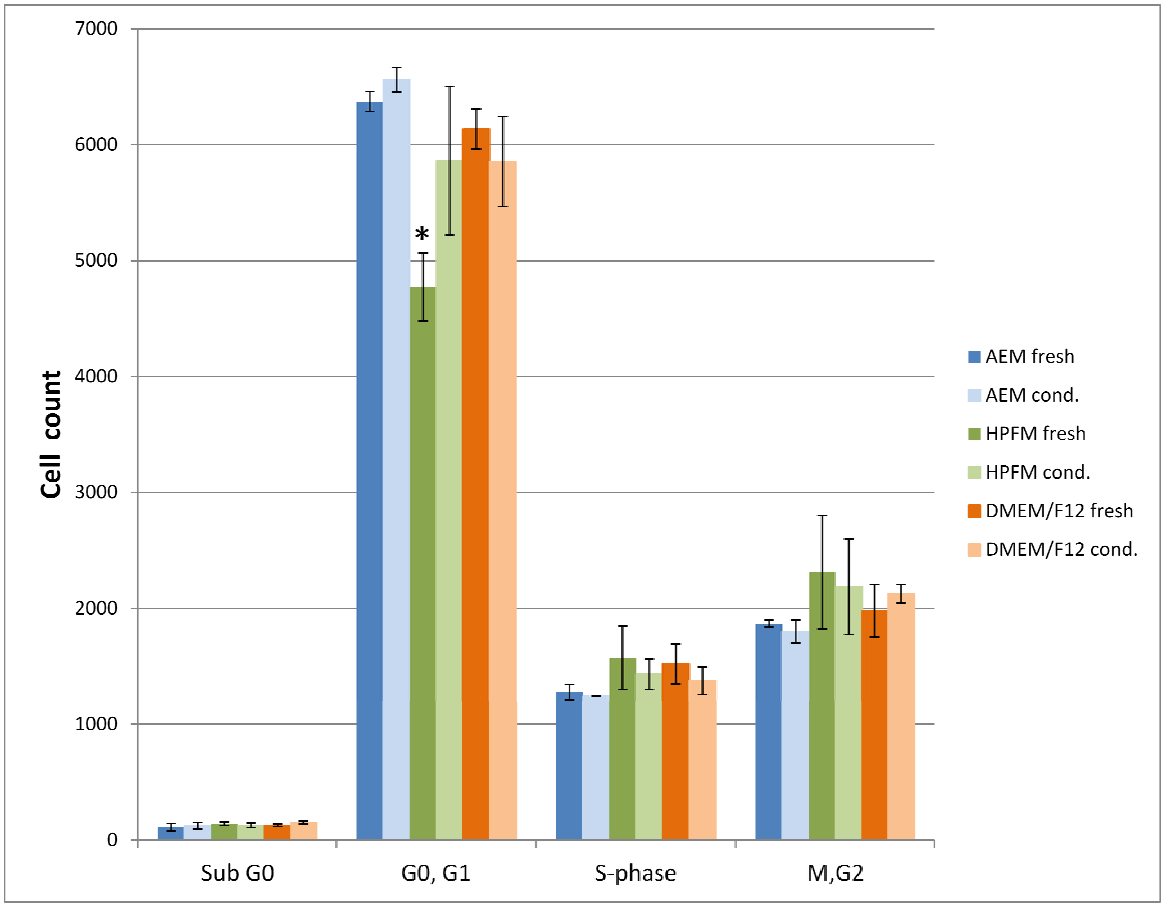

Figure 3.16 Cell cycle analysis of C38 after a 24 h exposure to AEM, HPFM and DMEM/F12 in fresh and conditioned form
For the epithelial cell line C38 no significant differences in the cell cycle profile could be identified for any of the used media apart from C38 being cultured in fresh HPFM. In this medium C38 show a significant decreased number of cells present in the G0/G1 phase and a slight increase in s-phase and M/G2 phase. C38 will therefore be cultured in their own specific AEM for all following experiments. Each bar represents the mean ± SD from three different experiments.

In figure 3.16 cell counts for C38 in different cell cycle phases are presented for each of the different media. In this case C38 show a significant decrease in the cell number presented in G0/G1 phase and additionally a slight increase in S-phase and M/G2 when cultured in fresh HPFM (dark green bars) instead of AEM.

3.4.4.3 Cell cycle analysis of IB3-1

As for the other two cell types HPF and C38, IB3-1 was used in the same way to identify which medium could serve as a suitable one for co-culture conditions. The table below (table 3.3) shows the typical cell distribution of IB3-1 when cultured in fresh AEM, which is very similar to the distribution of C38.

Cell cycle phase	Percent of gated cells	Std. Dev.
subG0	1.10%	0.29
G0,G1	61.40%	0.61
S-phase	12.80%	0.10
M,G2	23.20%	0.09

Table 3. 4 Normal distribution of IB3-1 cells across the cell cycle when cultured in fresh AEM

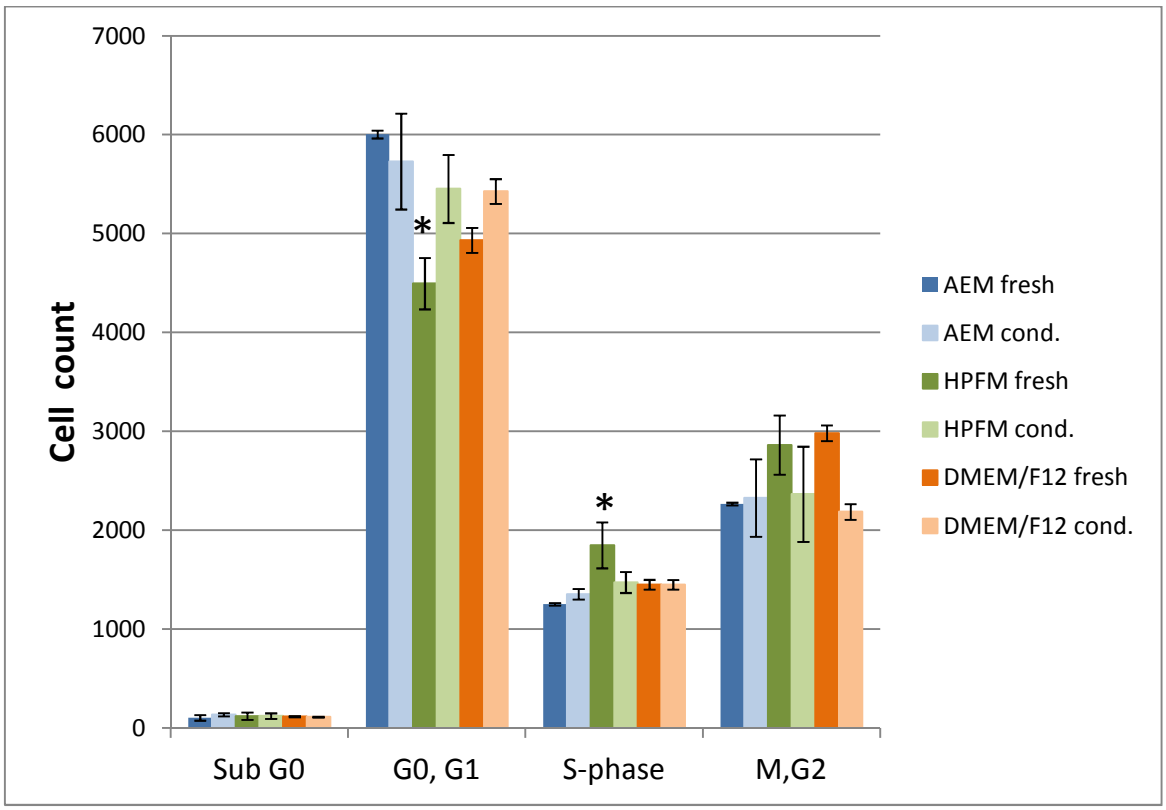

Figure 3.17 Cell cycle analysis of IB3-1 after a 24 h exposure to AEM, HPFM and DMEM/F12 in fresh and conditioned form
IB3-1's cell cycle profiles for all applied media were analysed and these cells do not show any significant differences in cell numbers being present in different phases of the cell cycle with the exception of IB3-1 cultured in fresh HPFM, where a significant lower number of cells is present in G0/G1 phase. In addition to this there is a significant increase in S-phase with more cells replicating, which is also reflected by an increase in M, G2. In this case the medium chosen for further experiments is AEM. Each bar represents the mean ± SD from three different experiments.

For IB3-1 the different cell cycle patterns shown in figure 3.17 reveal that culturing IB3-1 in fresh HPFM leads to a decrease of cell numbers in G0/G1 and to a significant increase in S-phase plus an increase in M/G2.

3.4.4.4 Cell cycle analysis of Calu-3

A standard cell cycle profile was produced for Calu-3 using fresh DMEM/F12, which is used here as their specific cell culture medium. The typical distribution of cells in the different phases of the cell cycle is shown in table 3.4.

Cell cycle phase	Percent of gated cells	Std. Dev.
subG0	3.80%	0.78
G0,G1	55.10%	2.98
S-phase	13.27%	2.39
M,G2	26.20%	1.98

Table 3.5 Normal distribution of Calu-3 cells across the cell cycle when cultured in fresh DMEM/F12

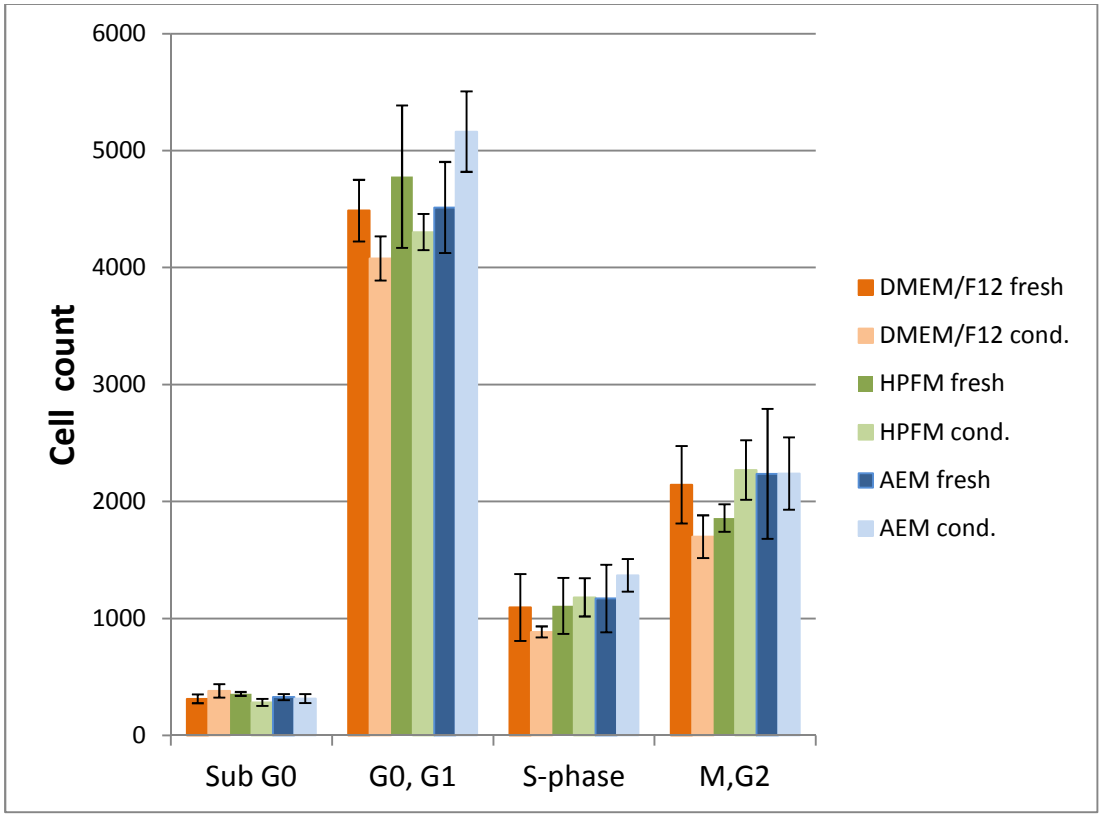

Figure 3.18 Cell cycle analysis of Calu-3 after a 24 h exposure to DMEM/F12, HPFM and AEM in fresh and conditioned form
Cell cycle profiles of Calu-3 cells were generated and analysed after these epithelial cells were grown in fresh and conditioned media (DMEM/F12, HPFM and AEM). In this case no significant differences of cell counts could be found for the different phases of the cell cycle. These cells will therefore be grown in their own specific DMEM/F12 medium for all mono- and co-culture experiments. Each bar represents the mean ± SD from three different experiments.

Looking at the distribution of Calu-3 cells counted in each of the cell cycle phases after being cultured in all the applied fresh and conditioned media (DMEM/F12, HPFM, and AEM), no significant increases or decreases could be observed.

3.5 Discussion

Placental human collagen type IV (collagen IV) was chosen as a suitable substrate for mono-culture and co-culture systems of human CF- and non-CF airways *in vitro* applying HPF, C38, IB3-1 and Calu-3 cells. An experiment was carried out to compare cell adherence on collagen IV to cell adherence on plain plastic tissue culture ware. The results clearly show that collagen IV supported cell adherence of all four different cell types investigated, as a higher number of cells adhered to collagen IV coated tissue culture ware in 24 h then to a simple uncoated plastic surface (figure 3.1). These findings fit together with results of other research groups that have been published. First of all it is known that collagen IV is one of the most abundant components in BMs, which underlies epithelial cells and so separates these from the stroma that lies underneath the BM (Sage, 1982). As epithelial cell interaction with the BM is essential for a normal and healthy development, they have receptors, that can be divided into integrins and non-integrin (e.g. heparan sulphate proteoglycans) receptors (Khoshnoodi *et al*., 2008). At least two of the integrins are collagen receptors and are constitutively expressed in the human adult lung *in vivo* as well as in cultured primary human airway epithelial cells *in vitro* (Wang *et al.*, 1996). Collagen IV is a network forming collagen that not only gives structural support to tissues and organs, it also has specific biological functions (Kadler *et al.*, 2007, Stanley *et al.*, 1982). One very important aspect that Fiedler *et al.* (1991) found is that human tracheal epithelial cells (HTE) cultured on a plastic surface lost the ability to produce an important protein called secretory component (SC), which is an important transport protein for sIgA. sIgA is usually found in the mucosal lining after it has been transported by SC across the cell and was released into the lumen of the airways, where it exhibits antimicrobial properties to protect the underlying epithelium. In addition to human airway epithelial cells and their need for collagen type IV to support cell growth, Kavvada *et al.* (2005) has found that guinea pig gastric epithelial cells need collagen IV to form a differentiated and polarized monolayer of cells by promoting tight junction (TJ) formation whereas collagen I did not support this to the same extent (Kavvada *et al.*, 2005). Furthermore it has been reported that other cell lines such as the Chinese hamster ovary cell line (CHO) and human fibrosarcoma cell line (HT-1080) showed distinct adherence to collagen IV (Aumailley and Timpl, 1986). Collagen IV sequencing revealed that certain domains in different isoforms of collagen IV show a high similarity and suggest that these are responsible for highly conserved functions across mammalian and invertebrate species (Than *et al.*, 2002). Collagen IV is now routinely used in airway related research to serve as a basement membrane and is commercially available from big biotechnological and chemical supplying companies.

Cell growth medium for *in vitro* cell culture supplies cells with defined nutrients and also contains essential growth factors, amino acids and vitamins, for example, which is vital for healthy cell growth and vitality. Because different cell types and cell lines have different nutritional requirements it is not always easy to have one medium to satisfy several different cell types. For the development of the co-culture model, however, one medium needed to be determined that can be used for a co-culture model that applies two different cell types, which are fibroblasts and epithelial cells, in the same system. One medium out of three (HPFM, AEM, DMEM/F12) that we routinely use for these cells, needed to serve for different combinations of fibroblasts and epithelial cells in the co-culture model. HPFM is the cell specific medium for HPF, AEM for C38 and IB3-1 and DMEM/F12 for Calu-3. The cell combinations for the co-culture model were HPF-C38, HPF-IB3-1 and HPF-Calu-3. All different cells have a typical morphology, which implies being flat, large and adherent for epithelial cells, depending on the origin of the cells and its specification as there are also different types of epithelial cells (e.g. serous, ciliated). Fibroblasts are, when adherent, long, flat and stretched in a spindle shape with an oval nucleus.
As described in chapter 1 the lower and upper airways display different cells and in different proportions depending on the region of the respiratory tract where they were found in but the entire respiratory system is lined by epithelial cells (Gail and Lenfant, 1983).

For the medium investigation all cells were cultured submerged to look for morphological changes (e.g. giant cell, multi-nucleated cells, or even ragged cell edges). Each cell type was cultured in each of the three media in fresh and conditioned form. To produce conditioned media, each cell type was grown in their cell specific medium, which was then collected for this experiment. Conditioned medium contains a different mix of leftover nutrients and everything that was secreted by the cells that were cultured in it, such as growth factors, cytokines and other proteins. These may have an effect on the other cells when cultured in it. The pictures of the light microscope of cell morphology (figure 3.3 – 3.6) showed that there was no sign of abnormal morphology for any of the cells analysed after they were cultured in one of the fresh or conditioned media (HPFM, AEM and DMEM/F12).

To look a bit further and in more detail if these media have an effect on the cells, a cell viability assay was performed to investigate if any medium was directly influencing the metabolic activity or if they are even cytotoxic to the cells tested. Furthermore a flow cytometric cell cycle analysis was carried out to investigate if the media on one hand could drive cells towards cell death or on the other hand promote proliferation.

The results for HPF showed that there is no significant difference in their metabolic activity when these were cultured in fresh or conditioned AEM compared to the metabolic activity when cultured in fresh HPFM (figure 3.15). This implies that they can be cultured in AEM as well as their specific HPFM, which is also true looking at the cell cycle profiles, as HPF do not show any significant shifts of cell numbers in the cell cycle (figure 3.3). However, for conditioned HPFM there is a significant increase in cell metabolic activity but again no difference can be observed when looking at the cell cycle profiles. For HPF another significant increase in metabolic cell activity was seen, when they were cultured in fresh DMEM/F12, compared to HPF cultured in fresh HPFM. There is one major difference between these two media. HPFM contains 11% FBS and DMEM/F12 contains 15% FBS as fresh media and this could be an explanation for the increase of metabolic activity. FBS is the most commonly used serum for tissue culture and the concentration usually varies from 5 – 20% (v/v) depending on the cells needs. The addition to the medium increases its quality as it contains basic nutrients, hormones, growth factors and binding proteins for example but the exact components that enhance cell growth are not known yet. Cells cannot survive without serum but too much is equally bad and it needs to be optimised for every cell line and primary cells if not already done so. To certain extend cell growth and proliferation can be stimulated by a higher concentration of FBS in the medium based on the rich supplements that come with it. This increasing metabolic activity was not observed when HPF were cultured in conditioned DMEM/F12. The FBS concentration in conditioned media will be less than in fresh media as some of its ingredients will have been used by cells grown in it before. These results of increasing metabolic activity when grown in conditioned HPFM and fresh DMEM/F12 were not exactly reflected in the cell cycle analysis. While the cell viability test is based on enzyme activity in the mitochondria and the cytosol to convert resazurin (non-fluorescent) into resorufin (fluorescent), the flow cytometric cell cycle analysis is based on staining the amount of DNA in each cell to be able to locate the stage of cell cycle that these cells were in.

Viable cells need to generate energy not only to enter and complete the cell cycle but also for the synthesis of essential components and for maintaining their membrane integrity, for example. This means that resazurin will eventually be enzymatically reduced to resorufin in their mitochondria or in the cytosol unless the cells are dead. Resting cells have a lower metabolic activity than proliferating cell but when cells come out of G0, which is a quiescent phase of the cell cycle, they need to increase transcription and translation to make proteins and enzymes that are necessary to enter the cell cycle again and to be able to fulfil the cell cycle. All together this could mean that we see an increase in metabolic activity of the cells preparing to enter the cell cycle actively again but as they are not synthesising DNA yet, this is not reflected in the cell cycle

profile by a shift of cell numbers towards S-phase and M/G2. This suggests that not all viable cells, that cause the metabolic activity increase, are proliferating at that time point. Another reason for the increase in metabolic activity could also be the confrontation of cells with growth factors, hormones and other ingredients in that medium that they were not confronted with in their own medium and therefore they have to produce enzymes for example to break them down or convert them. Again this initiates transcription and translation processes unless the needed enzymes have been readily available and this will increase the cells metabolic activity.
For C38 and IB3-1 the results are almost identical to each other with a significant increase in metabolic activity when cultured in conditioned HPFM or in either fresh or conditioned DMEM/F12. IB3-1 also showed a significant increase for metabolic activity when fresh HPFM was used for culturing them. This indicates a higher sensitivity of IB3-1 to environmentally changes.
As already mentioned before, conditioned media can have a stimulating effect on some cells, which could be down to certain cytokines and growth factors that have been secreted by the cells that were cultured in the medium before. To determine exactly what is causing this change in metabolic activity further detailed analyses of the media and all the contents would have to be performed. Fresh DMEM/F12 also increases the metabolic activity of C38 and IB3-1, which again could be down to the higher concentration of FBS, which is 5% in AEM (as recommended by ATCC) and 15% in fresh DMEM/F12. The higher concentration of FBS ultimately supplies cells with more growth factors and other supporting nutrients. Comparing these results to the cell cycle analysis results there were differences to note as flow cytometric analysis of C38 and IB3-1 only detected differences for fresh HPFM. There was a significant decrease of cell numbers in G1/G0 but simultaneously a slight increase of cell numbers in S-phase and M/G2 for C38. This increase in S-phase and M/G2 is actually significant for IB3-1. This again shows that IB3-1 react more sensitive to these medium changes. AEM was determined as the optimal medium for mono-and co-cultures that apply HPF and either of these two epithelial cell lines.

Calu-3 cells did not show any differences in cell viability for DMEM/F12 or AEM, which is consistent with the flow cytometric data. However, when cultured in HPFM, fresh or conditioned, these cells show a significant decrease in metabolic activity. Again this could be down to the lower concentration of FBS in HPFM plus of course the other ingredients in HPFM might not perfectly meet the needs of this cell line. These results are not directly reflected by the cell cycle analysis as there are no significant differences of cell cycle profiles compared to their baseline profile, when cultured in fresh DMEM/F12. Looking at the bar graph of the cell cycle analysis (figure 3.18) there is a slight trend towards more cells being in G0/G1, fewer cells in M/G2. Usually when cells encounter difficult times or dramatic changes they will leave the active

cell cycle and wind down metabolic activity while in G0 phase. So a decrease in metabolic activity does not necessarily mean that the cells are dying and that is why there is no increase in cell numbers in the SubG0 phase in this flow cytometric analysis as these cells still have intact membrane integrity and the DNA is not degraded.

Taken all together submerged cultures of HPF and mono-cultures of HPF at ALI were grown in HPFM throughout this project. For co-culturing HPF with either C38 or IB3-1, AEM was the chosen medium for all further experiments and epithelial cell mono-cultures of C38 and IB3-1the original culture medium AEM was used for submerged cultures. HPF-Calu-3 co-cultures were grown in DMEM/F12 as were the Calu-3 mono-cultures at ALI for further establishment and characterisation of these *in vitro* models for CF- and non-CF human airways.

4 Chapter 4 Characterisation of cell specific markers in the co-culture model at ALI

4.1 Introduction

In order to validate that the mono- and co culture models grown at ALI mimic the human airways *in vivo*, it was necessary to be able to identify the different cell types especially when grown in co-culture applying fibroblasts and epithelial cells in the same model system. After determining collagen IV was a supporting growth substrate and selecting suitable cell culture media for all *in vitro* models established, the challenge now was to select experimental methods that allow the identification of different cell types as well as visualizing their location and distribution in the model system. A further complexity was that the cells were to be grown on TWs at ALI in order to promote epithelial differentiation, which has been shown for several airway epithelial cell lines before (Wiesel *et al*., 1983, Gruenert *et al*., 1995).

Using light microscopy is not an option as it is almost impossible to see the cells once they are seeded on TWs and especially with the outlook towards an even more complex model employing HPF and epithelial cell lines, another method is needed. One way of identifying specific proteins, peptides and thereby different cell types is to use immunocytochemistry. In order to be able to identify an immunogen of interest, specific antibodies against that particular immunogen are raised and those can then be used in cultured cells or in tissues samples to detect localisation of the protein of interest (see 2.7). Many antibodies are commercially available from life sciences companies, such as Abcam.

All *in vitro* models presented in this project, apart from HPF in mono-culture, employ bronchial epithelial cells, which are known to express cytokeratins (CK). CKs are a subfamily of intermediate-sized filaments, are classed as epithelial cell specific markers. Expression profiles of CKs are not only cell type specific and tissue specific, but are also indicative of the cellular state of differentiation, which has been reported to be tightly correlated (Moll *et al*., 1982). CKs can be classed into type I (acidic) and type II (neutral basic), which always form heteropolymeric pairs with one another to constitute their filamentous structure (Moll *et al*., 1982). Type II CK5, for example, forms a heteropolymer with type I CK14 and is expressed in the basal epithelial layer, therefore antibodies to CK5 or CK14 are generally used to identify basal cells in epithelial tissue or cell cultures (Purkis *et al*., 1990, Moll *et al*., 1982). These intermediate filaments also serve basal cells to strongly attach to the BM through forming cell-ECM junctions, termed hemidesmosomes, supporting the mechanical integrity of the tissue. Additionally CK filaments

also span through the cytoplasm, hold the nucleus in place and attach to the desmosomes, intercellular junctions (Green and Jones, 1996).

As described in chapter 1, the large airways are lined by a pseudostratified epithelium (simple epithelium), which comprises not only basal epithelial cells, but also lumen-lining differentiated cells, which are columnar ciliated cells. These can be detected using antibodies against CK 8 or CK18, markers of differentiated epithelial cells, which are typically co-expressed to form the intermediate filament (Moll *et al*., 2008). Thus, antibodies to CK5 and CK8 could be used for presented mono- and co-cultures on TWs to specifically identify basal and differentiated epithelial cells respectively. In addition, a distinct, cell-specific marker was also needed for identifying fibroblasts in these mono- and co-cultures. For this, the 1B10 antibody against fibroblast surface protein and an antibody to vimentin were used. The 1B10 was developed in the nineties mainly to remove non-epithelial cells from thymic epithelial cell cultures, as it was shown that 1B10 binds human fibroblasts, tissue macrophages and peripheral monocytes (Singer *et al*., 1989). Vimentin is one of the main structural components of intermediate filaments expressed in cells of the mesenchyme. Intermediate filaments are expressed in fibroblasts and are needed not only for stability of the cell and structural support but also for some vital cell functions and motility; vimentin helps with chemotactic migration and it has been shown if vimentin is absent, fibroblasts show a slower wound-healing process (Eckes *et al*., 1998, Wang and Stamenovic, 2002, Eriksson *et al*., 2009).

Furthermore it was essential to analyse whether the mono- and especially the co-culture models mimicked the *in vivo* situation closely in terms of their appearance as a pseudostratified epithelium (with HPF underlining the epithelial cells), forming a confluent cell layer of tightly connected epithelial cells. Additionally, it was important to identify whether mucociliary ciliogenesis takes place and the expression of microvilli and cilia had been initiated. Cilia play an important role in the respiratory system as part of the innate defence mechanism by transporting the mucus and all entrapped bacteria, viruses and toxins towards the pharynx (mucociliary escalator). As described in chapter 1 this clearance and defence system is impaired in CF lungs because cilia get trapped in vast amounts of viscous and dehydrated mucus, which makes it impossible for them to beat and ultimately frustrates the mucociliary escalator (Houtmeyers *et al*., 1999). Thus, the evaluation of these novel *in vitro* mono- and co-cultures included an investigation for the presence of cilia and microvilli on the apical surface.

Another aspect of importance was the influence of HPF on pseudostratified mucociliary differentiation and intercellular communication. As already mentioned in chapter 3, mesenchymal- epithelial interactions as well as epithelial- ECM interactions are important for

lung development and repair (Knight, 2001). Furthermore fibroblasts play an active role in cytokine production for example (Kelley *et al*., 1991) as well as in growth factor production (Shoji *et al*., 1989).

In vivo the epithelial cells attach to a region called the basement membrane zone (BMZ), which serves as connective junction between these and the underlying connective tissue. The epithelium attaches through adhesins and hemidesmosomes to the BMZ and in close proximity to fibroblasts, which are immediately beneath the epithelium (Evans *et al*., 1993) and have been suggested to modulate several important functions, including recruitment and activation of inflammatory cells (Evans *et al*., 1993, Evans *et al*., 1999). The recruitment of neutrophils in the high numbers in which they appear in the CF airways has so far mostly been related to immune cell/epithelial IL-8 secretion whilst fibroblast involvement in the strong inflammatory response is still unknown.

There are co-culture models that have revealed that postmitotic fibroblasts, which serve as static feeder layers, are able to stimulate airway epithelial growth under submerged conditions and on TWs (Wiszniewski *et al*., 2006, Skibinski *et al*., 2007). However in these models the proliferation effects that epithelial cells have on fibroblasts were eliminated by irreversibly blocking proliferation of fibroblasts by using mitomycin C. Whether this has a direct effect on accumulation of epithelial derived stimulators, the direct physical interaction of these two cell types and the interaction with ECM are not known but these important features need to be investigated to elucidate the controlled interactions of these two cell types that are found *in vivo.* Furthermore it has been shown that mitomycin C upregulates IL-8 secretion in corneal fibroblasts, even after removal from culture medium (Chou *et al*., 2007). IL-8 is the key chemokine in CF and, since accurate analysis of IL-8 release is of paramount importance to this project, the use of mitomycin C-treated, IL-8 secreting fibroblasts would prevent this. In addition, the project aimed to measure the fibroblast response to stimuli, such as bacteria, therefore employing actively proliferating, responsive primary human fibroblasts is an imperative feature required to make these co-culture models more accurate and closer mimic the *in vivo* situation.

Additionally, proliferating HPF were in direct contact with the epithelial cells in the current model system without having a physical barrier (membrane) between the two. One challenge was thus to characterise the different cellular locations in the co-culture models and validate that active fibroblasts can be used for these kinds of studies.

For over twenty years mono-cultures of epithelial cells at ALI have been used routinely for studying epithelial cells and behaviour *in vitro.* During this time, better techniques have evolved

and biotechnological, semi-permeable membranes have improved but no one has reported a model applying proliferating, metabolically active airway fibroblasts directly underlying the epithelial cells, which are then grown at ALI. As already mentioned above, fibroblasts play an important role in lung development and cell-cell interactions (Shoji *et al*., 1989), therefore these models would be a valuable addition to existing cell culture models *in vitro* as well as to available animal models *in vivo*.

Further characterisation of the models, especially the co-culture models of non-CF and CF human airways *in vitro* is required to verify that the epithelium develops into a well differentiated functional pseudostratified epithelium in the mono- and co-culture model systems and secondary that the fibroblast cell population supports this and do not overgrow the epithelium.

4.2 Aims

The overall aim of the studies described in this chapter was to further characterise the *in vitro* mono- and co-culture model systems for non-CF and CF human airways with the criterion of epithelial differentiation in terms of generating a multi-cellular, ciliated and pseudostratified airway epithelium and to characterise the influence of HPF on epithelial differentiation in the co-culture model.

In order to achieve this, the first aim was to determine cell type specific markers useful for cell-specific immunocytochemistry labelling in mono-and co-cultures under submerged conditions as well as for cultures at ALI.

The second aim was to be able to distinguish between the epithelial cells and the fibroblasts and to localise their position, especially in the co-culture models. In order to achieve this histologic analysis of mono- and co-culture cross sections (ALI) was performed.

The third aim of this chapter was further investigation of fibroblast and epithelial cell layer morphology in mono-and co-cultures and to investigate whether cilia formation takes place and whether this is modulated by HPF as subepithelial cell layer.

4.3 Methods

4.3.1 Antibodies

Mouse monoclonal antibody to fibroblast surface protein (1B10; ab11333, 0,2 mg/ml), mouse monoclonal anti-cytokeratin 8 antibody (0.5 mg/ml, ab9023) and mouse monoclonal anti-basal cell cytokeratin (CK5) antibody (1 mg/ml ab9272) were purchased from Abcam (Cambridge, USA). The mouse monoclonal anti-vimentin [V9] antibody was purchased from Gene Tex (GTX76575), mouse IgG kappa (Mopc 21, 5 mg, m-7894), and goat anti-mouse fluorescein isothio-cyanate (FITC) secondary antibody (F0257) was purchased from Sigma.

4.3.2 Immunocytochemistry

Immunocytochemistry was performed on 4- well slides as well as TWs and the methods are described in detail in chapter 2.7.2 and 2.7.3. All primary antibodies used were mouse monoclonal antibodies, as detailed above. The secondary antibody for all staining was goat anti-mouse fluorescein isothio-cyanate (FITC, green). The staining was always finished by mounting the samples in a hard set mounting medium containing DAPI to stain the nuclei blue.

4.3.3 Histology

The TW samples were fixed with 10% (v/v) formaldehyde added to the cell culture medium. Any further processing, sectioning and staining was performed in Birmingham University by Gary Reynolds, Healthcare Scientist. The process is described in more detail in chapter 2.8.

4.3.4 Electron microscopy

Electron microscopy was conducted at the University of Birmingham at the Department of Metallurgy with the expert assistance of Paul Stanley (Manager of the Centre for Electron Microscopy). His protocols for SEM and TEM are described briefly in chapter 2.12 .

4.4 Results

4.4.1 Immunocytochemical characterisation

To determine the presence and distribution of the two different cell types in the final establishment of the proposed co-culture model system, different antibodies needed to be employed that were cell type specific (i.e. fibroblast or epithelial) and did not show any reactions with the other cell types.

4.4.1.1 Immunocytochemistry on submerged cell cultures on 4-well slides

In order to identify whether the chosen antibodies suited the requirements in terms of being able to differentiate between epithelial cells and fibroblasts in the final co-culture model, immunocytochemical staining was performed on submerged cell mono-cultures. This provided a first indicator of the specificity, and therefore usefulness, of these antibodies (described in detail in 2.7.2).

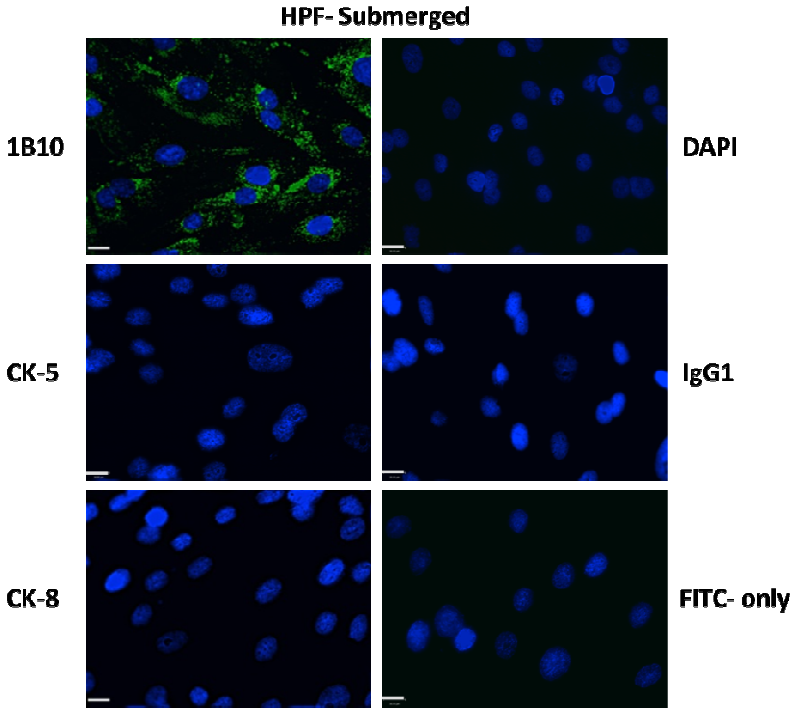

Figure 4.1 Representative immunofluorescence images of submerged cultures of HPF
HPF cells were seeded on collagen IV coated 4 well slides at a density of 3.5 x 10^4 cells/well in 200 µl medium and were incubated for 2 days before immunocytochemistry staining was performed. 1B10, an antibody against fibroblast surface antigen stained HPF positive (green), whereas the two antibodies against the epithelial cell markers CK5 and CK8 did not show any staining. Alongside with the antibodies for cell type specific markers, staining with IgG1 isotype control and secondary antibody on its own was performed, which were all negative. One sample was fixed and stained with DAPI only, which was also used as counterstain in all other samples. Images are representative of three individual experiments each done in duplicates and random fields of view were chosen and images taken (x 63 magnification; scale bar = 31µm)

After HPF were cultured for 2 days under submerged conditions on 4-well slides they were fixed and stained with 1B10, which binds to a fibroblast surface protein. The other two antibodies used for characterising epithelial cells were anti-cytokeratin antibodies. In this experiment we used antibodies to CK5 (basal cells) and CK8 (lumen lining). In addition we performed the whole experiment following the same protocol with an isotype control (IgG1) or with using only the secondary antibody goat anti-mouse FITC. One other additional staining was done with DAPI only but this sample was only fixed before stained in order to see if processing has a significant impact on cellular morphology. Representative fluorescent images are shown in figure 4.1 for submerged fibroblasts. HPF show positive staining for the fibroblast surface antigen (green) and DAPI stained nuclei (blue). No antibody specific staining was observed for CK5 and CK8 in submerged HPF cell cultures. The two controls using IgG1 as isotype control or the secondary antibody on its own were negative for detectable FITC signal. DAPI staining in the absence of antibody treatment showed the same nuclear appearance as on all other samples.

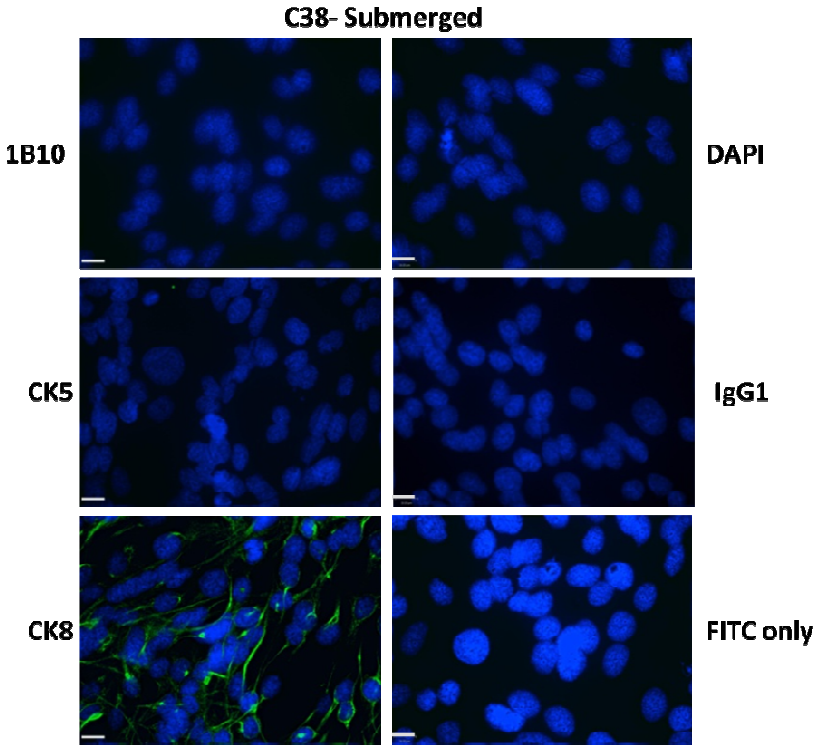

Figure 4.2 Representative Immunocyotchemistry staining of submerged C38
C38 were seeded on collagen IV coated 4 well slides at a density of 3.5 x 10^4 cells/well in 200 µl medium and after a 2 day incubation time, immunocytochemistry staining was performed. It appeared that 1B10, an antibody against fibroblast surface antigen did not bind to the cell surface of C38, since no detectable fluorescent signal was seen. The epithelial cell marker CK5 did not show any specific binding to this cell line either but when using CK8 a positive staining result was observed. Alongside cell marker antibodies, three negative control staining procedures were carried out: DAPI alone, IgG1 isotype control alone and secondary antibody alone (FITC only). Images are representative of three individual experiments each done in duplicates and fields of view captured at random for presentation (x 63 magnification; scale bar = 31µm)

Submerged C38 cells were cultured on collagen IV coated 4-well slides for two days before antibody staining was carried out. For the fibroblast marker antibody 1B10, C38 were found to be negative and only DAPI stained nuclei are visualized (figure 4.2 top left panel). CK5, which is a basal epithelial cell marker, was noted to be absent from C38 cells as well. However, there is strong positive staining for CK8 and this marker can therefore be used to identify epithelial cells in these mono- and co-culture models. C38 are also negative for the two controls and as for HPF, DAPI showed that processing after fixation does not change cell morphology.

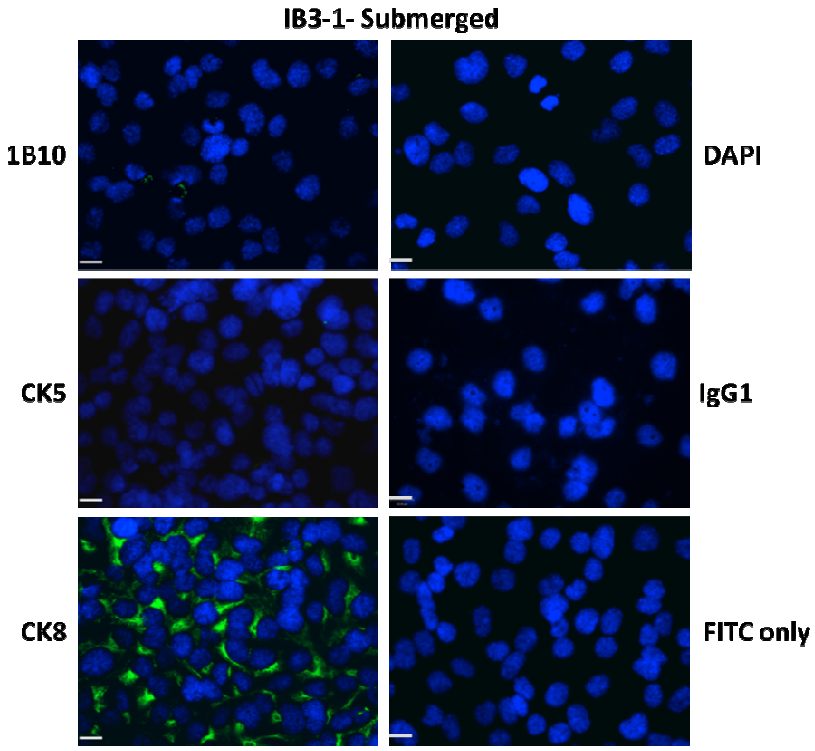

Figure 4.3 Representative immunocytochemistry staining of the epithelial cell line IB3-1
IB3-1 were seeded at a density of 3.5 x 10^4 cells/well in 200 µl medium on collagen IV coated 4 well slides and were incubated for 2 days before immunocytochemistry staining was performed. 1B10, an antibody for fibroblast surface antigen did not specifically bind to the cell surface but a low signal of fluorescence could be detected on some cells. The epithelial cell marker CK5 did not show any specific binding to this cell line, like C38, but when using CK8 a positive staining result is observed. Additionally to the cell marker antibodies, staining with IgG1 isotype control and secondary antibody on its own were performed, which were all negative. One sample was stained directly after fixing with DAPI only, which was also the counterstain in every sample shown. Images are representative of three individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm).

Immunocytochemistry staining of IB3-1 revealed that the epithelial cell marker CK8 is strongly expressed in these cells, whereas, whilst the anti-CK5 antibody does occasionally show reaction with this epithelial cell line, the staining intensity is very weak. 1B10 is the antibody against

fibroblast surface antigen and in some cases a very weak fluorescence signal on some cells could be detected. Cells stained with DAPI alone showed that nuclei look round and equally stained with no morphological changes to be identified in the other samples through processing (figure 4.3).

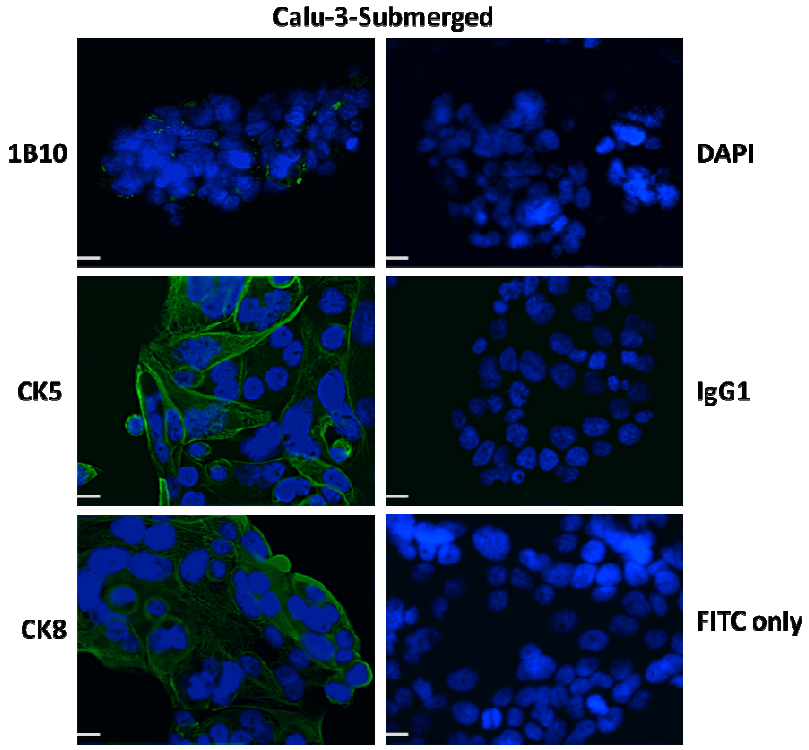

Figure 4.4 Immunocytochemistry staining representative of Calu-3 cells in submerged culture
Calu-3 cells were seeded at a density of 3.5 x 10^4 cells/well in 200 µl medium on collagen IV coated 4 well slides and after an incubation of 2 days immunocytochemistry staining was performed. 1B10, an antibody used as fibroblast marker did show some binding to the cell surface of Calu-3. The epithelial cell marker CK5 and CK8 both stain Calu-3 specifically. The counterstain DAPI was used in each sample plus on its own directly after fixation to verify that processing does not change cell morphology. An IgG1 isotype control and secondary antibody only, was performed, which were all negative. Images are representative of three individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

In figure 4.4 results for immunocytochemistry staining of Calu-3 cells is presented. The antibody 1B10, which was used as fibroblast marker antibody, appears to recognise the cell surface of a population of the Calu-3 cells, but this staining is extremely weak compared to expression of the epithelial cell markers. In contrast to the other two cell lines Calu-3 also stains strongly positive for the basal epithelial cell marker CK5. The green fluorescence created by the antibody staining visualizes the intermediate filaments clearly and seems to be located around every nucleus. Some cells seem to be slightly on top of others as they are clearly shown in their full outline. The anti-CK8 antibody for differentiated lumenal epithelial cells also shows strong staining of these intermediate filaments, which seem to be denser then CK5. The negative control of IgG1 and

secondary antibody on its own was negative for any green fluorescence due to antibody binding. Again, DAPI staining applied straight after fixing Calu-3 cells, showed that fixation and processing has had no direct effects on cell morphology.

4.4.1.2 Immunocytochemistry staining of HPF mono-cultures grown in submerged culture on TWs and epithelial cell mono-cultures on TW at ALI

Additionally to immunocytochemistry staining performed on 4-well slides, it was necessary to perform the same staining protocol for HPF grown in submerged culture on TWs as well as the epithelial cell lines grown at ALI on TWs to analyse if the different growth conditions and the differentiation of epithelial cells would change the staining pattern of each cell type compared to cells grown in complete submerged conditions. For this experiment HPF were grown on TWs but this cell type has to be kept submerged, whereas the different epithelial cell lines were put at ALI after 4 days submerged culture on TWs (see 2.4.6 and 2.7.3). After a further 14 days of culture the cells were fixed, permeabilised and blocked before the primary antibodies 1B10, CK5 and CK8 were applied. After the defined incubation time the secondary antibody was applied to all samples apart from one TW per cell type, which was pulled out the processing line earlier; straight after fixation one TW was stained with DAPI only again to compare if processing changes the morphological appearance of the cell layer. This was done for every cell line and as there were no differences to be noted these images will not be discussed for every figure but will be presented.

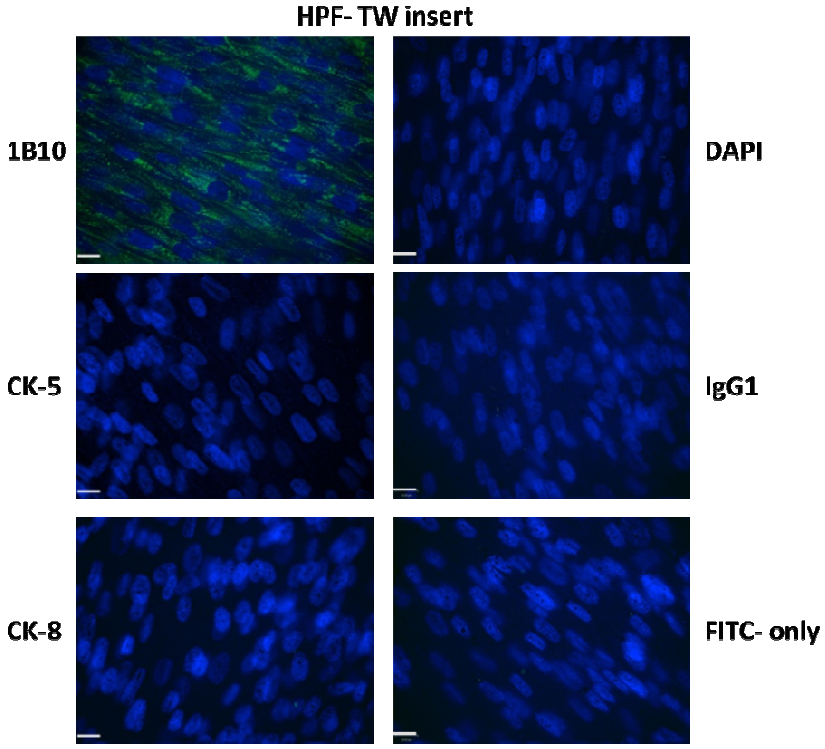

Figure 4.5 Immunocytochemistry pictures of HPF cultured under submerged conditions on TWs
HPF cells were seeded on collagen IV coated TWs at a cell density of 3 x 10^4 cells/well. After 14 days in submerged culture HPF mono-cultures were immunostained with 1B10, which gives the only positive staining, whereas CK5 and CK8 do not stain HPF. Negative controls of IgG1 and secondary antibody only were run alongside. Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

HPF grown under submerged conditions on collagen IV coated TWs were immunostained using the same antibodies as were used to stain cells under submerged conditions on 4 well slides. For HPF the same staining pattern was observed as was seen for submerged cultures. The fibroblast marker antibody 1B10 showed specific staining of HPF and clearly delineated the spindle-like morphology of this cell type, whereas the two other antibodies against epithelial cell markers do not show any specific staining under these culturing conditions. The controls were all negative indicating specific staining of HPF.

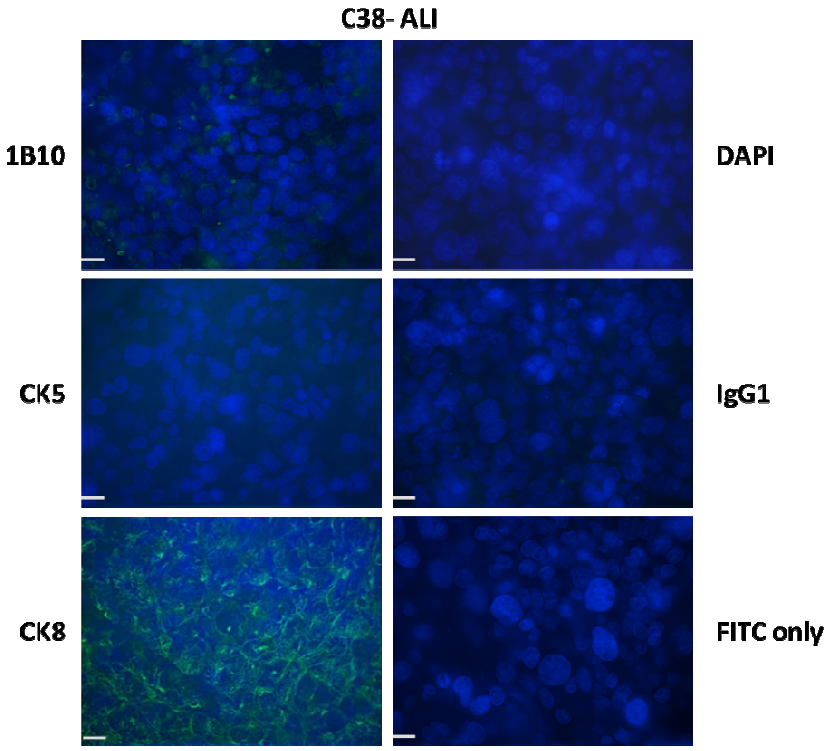

Figure 4.6 Immunocytochemistry pictures of C38 mono-cultures at ALI
C38 were seeded on collagen IV coated TWs at a cell density of 3 x 10^4 cells/well. After 14 days in culture at ALI the mono-cultures were immunostained with 1B10, CK5 and CK8 as primary antibodies followed by goat anti- mouse FITC as secondary antibody. For 1B10, a little positive staining of C38 was observed. CK5 does not stain these cells but CK8 shows positive staining. Negative controls of IgG1 and secondary antibody only were run alongside. Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

The staining patterns of the two epithelial cell lines C38 (figure 4.6) and IB3-1 (figure 4.7) differ from what was seen for these cells in submerged cultures. For both cell lines grown at ALI, positive staining was seen with the 1B10 antibody, which is directed against a surface protein on fibroblasts. Moreover C38 was negative for CK5 under submerged conditions and still is when grown at ALI but IB3-1 showed weak positive staining for CK5 when cultured at ALI. Strong CK8 staining in both mono-cultures of C38 and IB3-1 grown at ALI was observed.

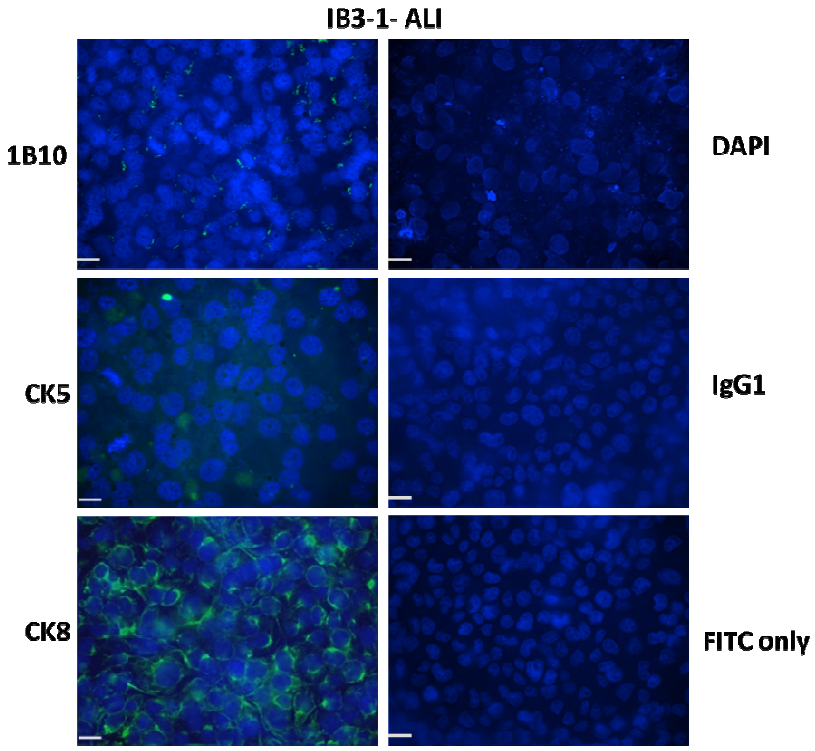

Figure 4.7 Immunocytochemistry pictures of IB3-1 mono-cultures at ALI
IB3-1 were seeded on collagen IV coated TWs at a cell density of 3 x 10^4 cells/well. After 14 days the mono-cultures were immunostained with 1B10, which showed very little positive staining, with CK5, which also shows little staining and CK8 as the differentiated epithelial cell marker showing strong specific staining. Negative controls of IgG1 and secondary antibody only were run alongside. Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

The Calu-3 cell line grown at ALI (figure 4.8) showed the following staining patterns after being grown in mono-culture at ALI for 14 days.

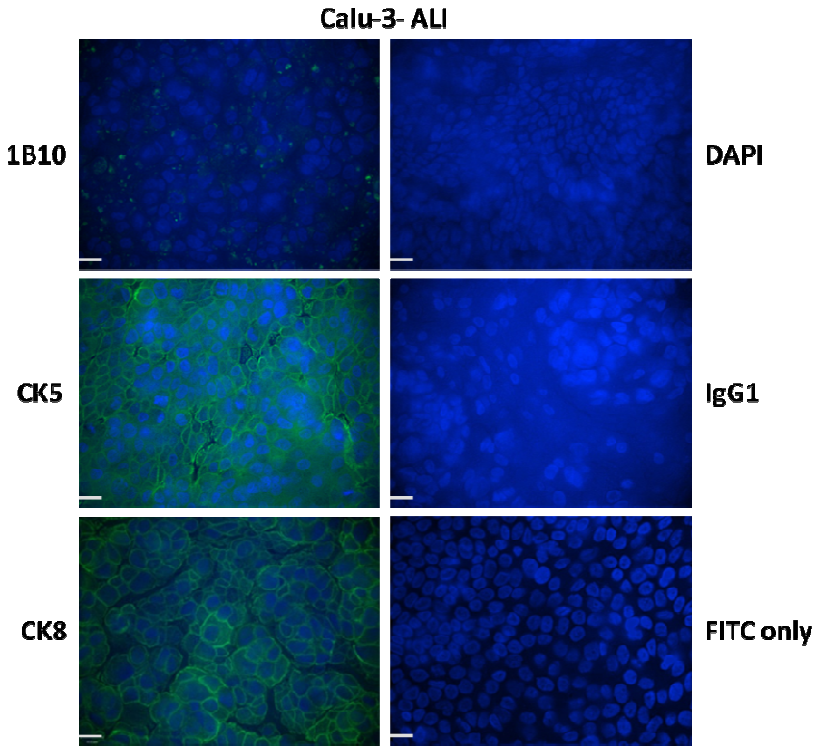

Figure 4.8 Immunocytochemistry pictures of Calu-3 mono-cultures at ALI
Calu-3 cells were seeded on collagen IV coated TWs and after 14 days in culture at ALI the mono-cultures were immunostained with 1B10, which was observed to show little staining. Antibodies against the epithelial cell markers CK5 and CK8 both showed strong specific staining of these cells under these conditions. Negative controls of IgG1 and secondary antibody only were run alongside. Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

1B10 immunostaining revealed a positive result for these cultures showing 1B10 staining around some Calu-3 cells presented in the image but no particular pattern can be identified. Strong staining of CK5 and CK8 was seen in these ALI cultures of Calu-3.

All negative controls that were run along with each of these experiments showed no staining, indicating that other stainings were specific for of any of the cell types. DAPI staining was carried out on one TW per staining procedure straight after fixation of cells to stain the nucleus, which was then compared to all other staining to be able to identify possible changes of cell morphology due to processing. The anti-cytokeratin antibodies were observed to be cell type specific and stain only epithelial cells, whereas 1B10 appears to show very weak cross-reaction with all three epithelial cells when grown at ALI.

4.4.1.3 Immunocytochemistry staining of co-cultures grown at ALI

The establishment of co-culture models applying HPF and one of the epithelial cell lines C38, IB3-1 or Calu-3 needed to be analysed for cell type location and distribution throughout the model. HPF were seeded first into the collagen IV coated TW and incubated for four days before the epithelial cells were seeded on top. Further incubation under submerged conditions was allowed for the epithelial cells to adhere and proliferate before establishing ALI.

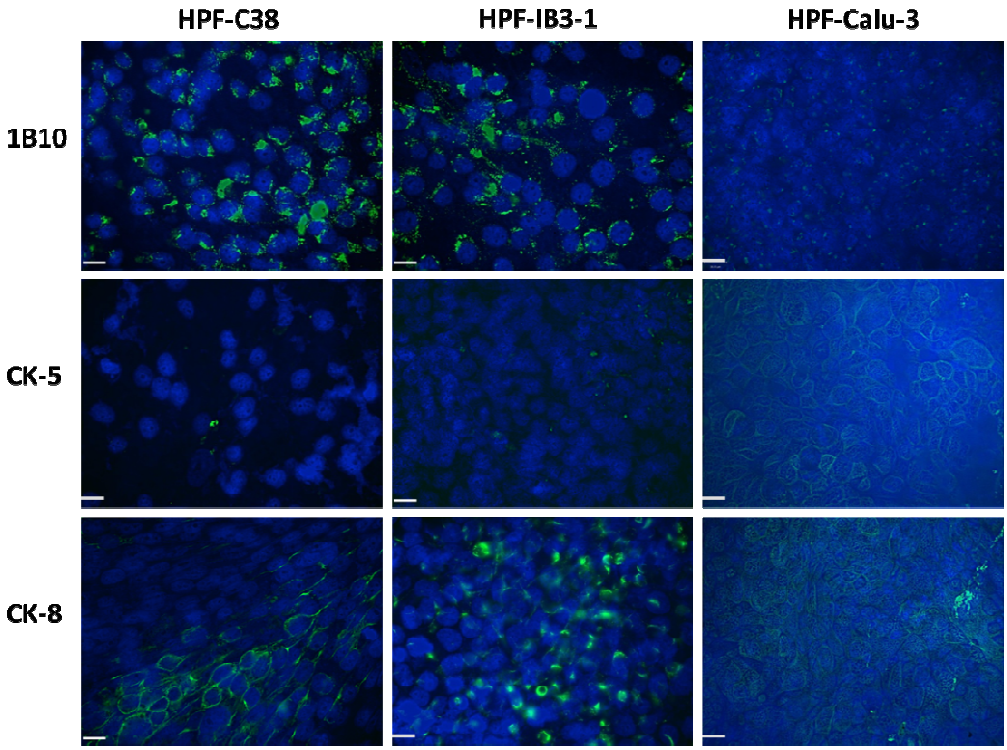

Figure 4.9 Immunocytochemistry staining of co-cultures at ALI (HPF-C38, HPF-IB3-1, and HPF-Calu-3)
After 14 days at ALI the co-cultures were immunostained with the same antibodies as the mono-cultures. These were 1B10, CK5 and CK8. 1B10 staining was observed in all three co-culture systems but much stronger in HPF-C38 and HPF-IB3-1, whereas these 2 models show very weak CK 5 expression compared to HPF-Calu-3, which show weak 1B10 staining but strong CK5 staining. All three co-cultures show strong expression of CK8 but the appearance of staining pattern is different in all three of them different. Negative controls of IgG1 and secondary antibody only were run alongside as well as one sample being stained with DAPI directly after fixing the cells for observation of morphological changes through processing (data not shown). DAPI also served as counterstain for all TWs analysed (blue nucleus). Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm)

Immunocytochemistry staining of all three co-culture model systems was undertaken and showed that expression patterns of the different immunogens for these antibodies are the same as for mono-cultures grown at ALI. 1B10 showed positive staining in all co-cultures but to a much

lesser degree in the HPF-Calu-3 model system, whereas the epithelial cell markers CK5 and CK8 were both strongly expressed in HPF-Calu-3. However, CK5 just showed very weak staining in the other two models. CK8 is strongly expressed in HPF-C38 and in HPF-IB3-1 but it appeared to show a slightly different pattern when comparing to each other. Compared to each other HPF-C38 and HPF-IB3-1 showed very similar staining for 1B10 but this reaction indicates that this is not a possible method to distinguish between the two cell types for any of these co-culture models.

Immunocytochemistry is a useful tool for identifying expression profiles of cell type specific markers, such as cytokeratins for epithelial cells, in mono-cultures under submerged conditions as well as for mono-cultures at ALI. However, antibodies can sometimes show non-specific staining or cross-reaction with different antigens on other cell types than those proposed by the manufacturer. This appears to be the case for the 1B10 antibody, which showed not only staining of fibroblasts but also some staining of all applied epithelial cultures (apart from C38 submerged) and can in this case not be used for further characterisation of the co-culture system for distinguishing the two different cell types in these model systems.

For this reason another fibroblast antigen vimentin, was selected and tested. The vimentin antibody was shown to be specific for fibroblasts without showing any reaction with the epithelial cell surface of C38, IB3-1 or Calu-3 (figure 4.10).

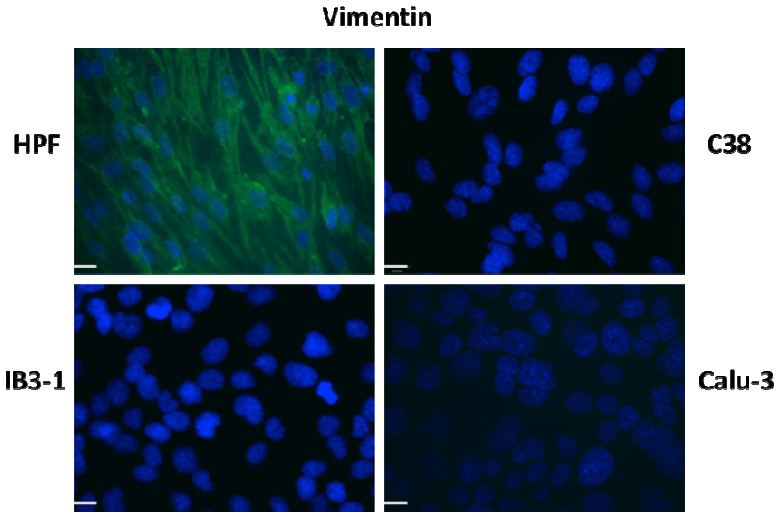

Figure 4.10 Representative immunofluorescent images of Vimentin staining on HPF, C38, IB3-1 and Calu-3 submerged mono-cultures on 4-well slides.
Cells were seeded on collagen IV coated 4 well slides at a density of 3.5 x 10^4 cells/well in 200 µl medium and were incubated for 2 days before immunocytochemistry staining was performed. Vimentin, an antibody targeting intermediate filaments in fibroblasts was used as primary antibody and was visualized using a FITC labelled secondary antibody. Positive staining could only be indentified for HPF and not for any of the epithelial cell lines. The negative controls, IgG1 isotype control and secondary antibody only as well as DAPI on its own are shown figure 4.1- 4.2. Images are representative of two individual experiments each done in duplicates and random pictures were chosen (x 63 magnification; scale bar = 31µm).

Figure 4.10 shows that the anti-vimentin antibody showed absolute specificity for fibroblasts, when observing HPF and the three bronchial epithelial cell lines. Positive fluorescent staining was only detected on fibroblasts (indicating antibody binding) while the epithelial cells were only visible because of their DAPI-stained nucleus. Therefore, the anti-vimentin antibody appears to make it possible to accurately, specifically distinguish between fibroblasts and epithelial cells. As fluorescent microscopy, where the cultures are viewed from the top (epithelial to fibroblasts to membrane), can be useful as shown above, cross sectioning mono- and co-cultures for distinguishing the location of fibroblasts and epithelial cells followed by histoloigic analysis appeared to more clearly reveal where cell are in the model. For this method vimentin will be used for fibroblasts.

4.4.2 Histological staining of mono- and co-culture models at ALI

Histology processing was performed at Birmingham University after mono- and co-cultures were formalin fixed in our laboratory.

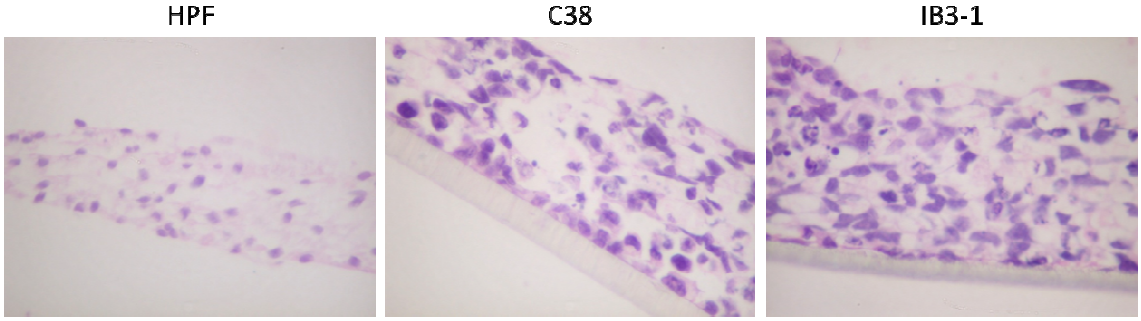

Figure 4.11 Light micrographs of hematoxylin and eosin (H&E) stained cross sections of mono-cultures of HPF (submerged but on TW) and C38 and IB3-1 grown at ALI.
Hematoxylin stained the nuclei, while eosin was used to counterstain the non-nuclear tissue components and cytoplasm. In all three cell types HPF, C38 and IB3-1 the dark purple nuclei could be identified readily. HPF showed a much greater distance between the nuclei compared to the epithelial cells, as their spindle shaped cell bodies were stretched out, whilst the epithelial mono- cultures both show a differentiated cell population, in which cells are closely linked together to form a pseudostratified like epithelium with much less intercellular space compared to HPF. No gross histological differences could be determined between the non-CF and CF model. The membrane of the TW was accidently separated from the HPF section, whilst it can still be seen on pictures of C38 and IB3-1. Images are representative of two TWs processed for each cell type.

Histological staining on paraffin embedded cross sections of mono- and co-cultures was performed as earlier described in 2.8. Formalin fixed cross sections shown in figure 4.11 were haematoxylin and eosin (H&E) stained to visualize nuclei (dark purple) and the cytoplasm (pink)

of the three mono-culture models. On the image of HPF mono-culture, the TW membrane has been lost during processing, whereas for the cross sections of C38 and IB3-1 the TW membranes can clearly be seen underneath the cells. The image in the left panel shows that the HPF grow as multiple, disorganised cell layers showing their typical long and spindle shaped morphology. The oval elongated nuclei are surrounded by a large stretched out cytoplasm that was stained pink and it looks like these cells are rather "gappy" compared to the tight appearance of epithelial cells. For epithelial cell lines this is quite a different picture, as the epithelial cell lines C38 and IB3-1 (middle and right images respectively) have built up to a pseudostratified epithelium with little pink stained cytoplasm compared to the HPF. For the epithelial cells, the nuclei are quite close but located on different levels, which is a hallmark of pseudostratified epithelia. Interestingly, no obvious morphological differences were seen between C38 and IB3-1. After the mono-culture cross sections were assessed, the co-culture systems were analysed using vimentin staining to locate the fibroblasts.

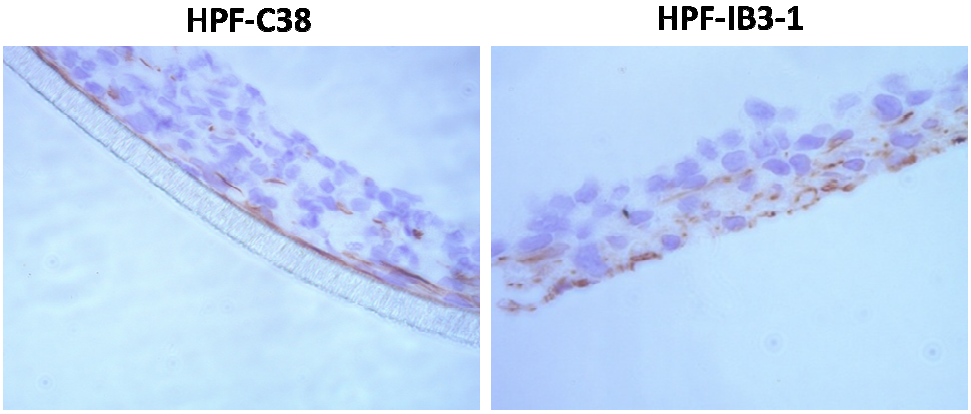

Figure 4.12 Formalin fixed cross sections of co-cultures of HPF-C38 and HPF-IB3-1 stained with H & E and Vimentin to identify the fibroblast's location in the model system. HPF-C38 showed a pseudostratified like epithelium formation with vimentin stained fibroblasts underneath the epithelial cell layer. The same was observed for HPF-IB3-1 but unfortunately the membrane of the TW was lost during processing. It also appears that the epithelial cell layer on top of the HPF is a bit smaller for the IB3-1 co-culture model (n=2).

The cross sections of HPF-C38 and HPF-IB3-1 showed that the introduction of fibroblasts into the co-culture model system does not appear to inhibit the epithelium from forming a tall, multilayered epithelium that appears to be pseudostratified with nuclei being located at different levels throughout, which resembles the appearance of the respiratory epithelium. The vimentin-positive HPF are mainly located underneath the epithelial cell layer.

4.4.3 Scanning electron microscopy of mono- and co-cultures grown at ALI

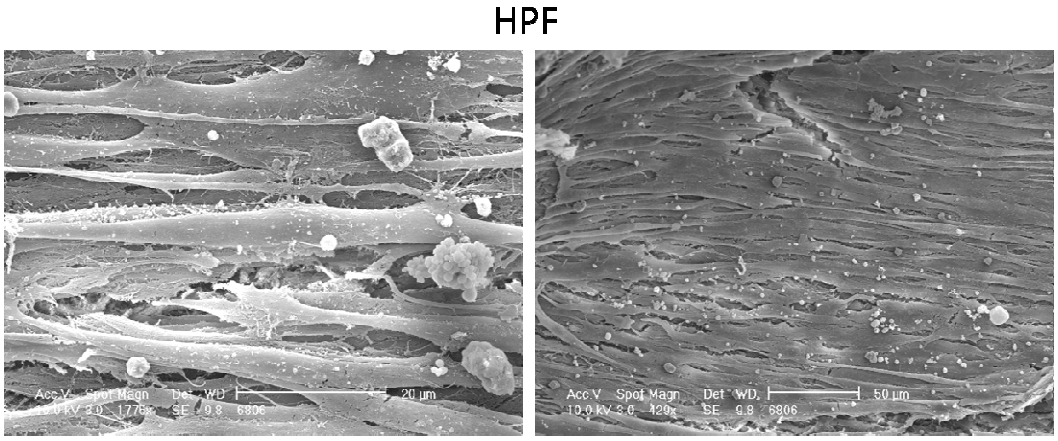

Figure 4.13 Scanning electron micrograph of HPF mono-culture
HPF showed their typical morphology after being cultured at least 14 on a TW under submerged conditions. This appearance implies a long and spindle shaped cell (left picture) that stands in close contact with neighbouring cells (right picture) forming a confluent cell layer. This layer of cells looked like one big sheet of fibroblasts. Images are representatives of two individual experiments. Scale bar, left picture = 20 µm; right picture = 50 µm

When HPF were grown in mono-culture on TWs under submerged conditions, scanning electron microscopy revealed that they formed a confluent layer of spindle-shaped cells (figure 4.13).

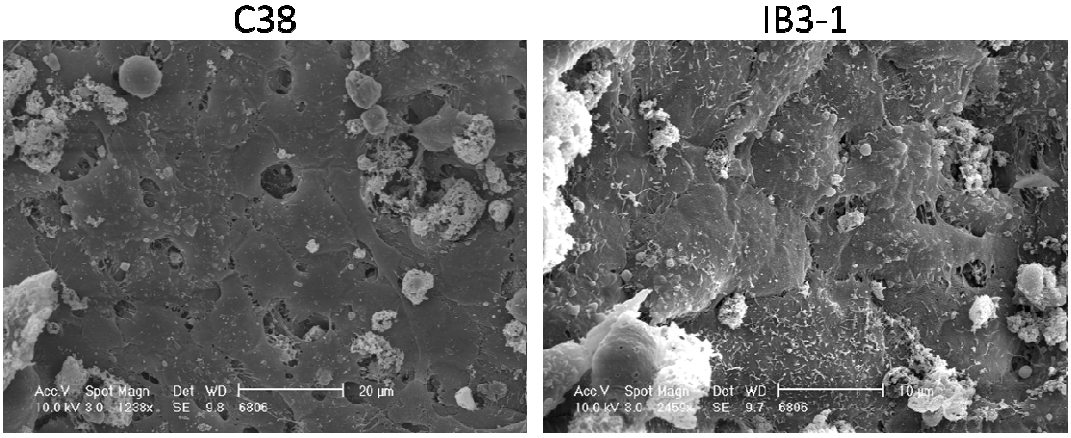

Figure 4.14 Scanning electron micrographs of C38 and IB3-1 mono-cultures at ALI
Both epithelial cell lines form a confluent and tight layer of cells when cultured on TW at ALI for at least 14 days. Microvilli are expressed on the apical surface of both cell lines but they seemed to be more numerous on IB3-1 cells. Cell morphology of both cell lines is the same; they appear as large, flat cells, which appear closely connected to neighbouring cells. Scale bar: C38 = 20 µm; IB3-1 = 10 µm (n=4)

SEM analysis (figure 4.14) revealed that C38 and IB3-1 mono-cultures at ALI grow as a confluent layer of cells with epithelial cell morphology, close connections to their neighbouring cells Furthermore microvilli expression was observed in mono-culture on the apical surface of both epithelial cell lines, although these microvilli and immature cilia were unorganised and differed in length. One difference between C38 and IB3-1 mono-cultures was that the microvilli seemed to be more numerous on IB3-1 mono-cultures.

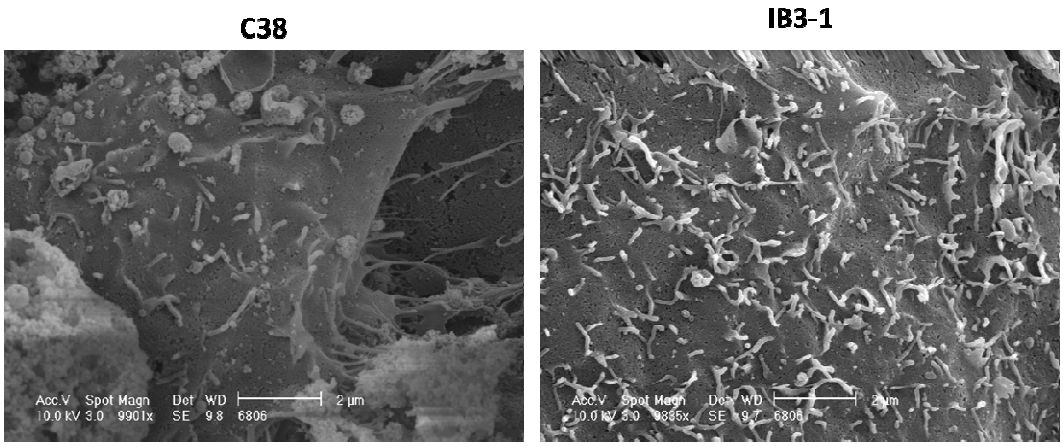

Figure 4.15 Scanning electron micrographs of C38 and IB3-1 mono-cultures' microvilli expression
Mono-cultures of C38 and IB3-1 showed heterogeneous expression of apical extensions and some short microvilli and some longer ones, possibly immature cilia. The number of microvilli seems to be higher on IB3-1's apical surface. Scale bars in both pictures are 2 µm.

A close up of the apical surface of C38 and IB3-1 mono-cultures showed the heterogeneous microvilli expression in more detail and supports the suggestion that IB3-1 express more microvilli. The expressed microvilli are about 1 µm long pointing in several directions and did not show any kind of organization (figure 4.15).

Co-cultures of HPF-C38 and HPF-IB3-1 were also analysed using SEM and these cultures showed confluent cell layer with clear cell-cell boundaries (figure 4.16). In addition these SEM images of the upper-most apical side of the co-culture show that the cells presented at the uppermost surface were epithelial cells and that the HPF cell layer is located subepithelial and therefore were not visible, which was already shown earlier by H & E and vimentin staining of paraffin-embedded sections of co-cultures (figure 4.12).

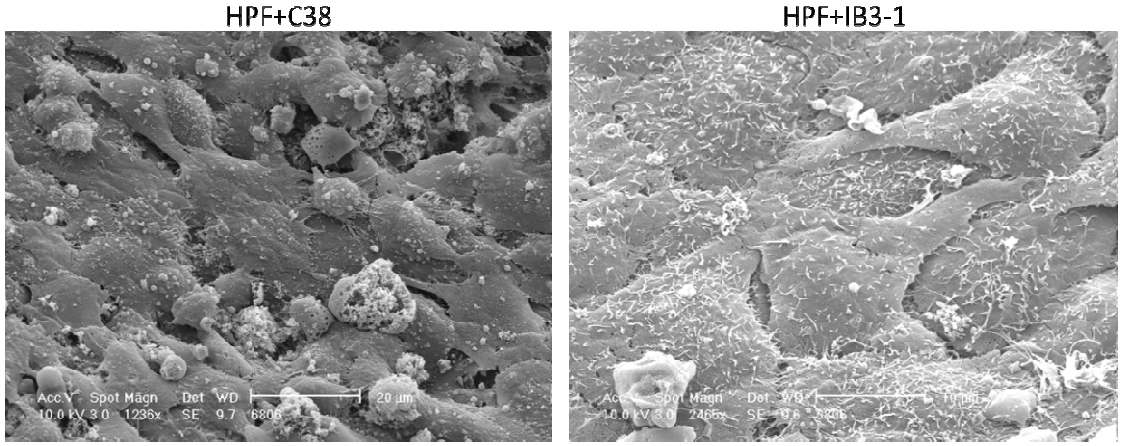

Figure 4.16 Scanning electron micrographs of HPF-C38 and HPF-IB3-1 co-cultures at ALI
SEM analysis of fibroblast and epithelial cell co-cultures showed a confluent and tight layer of ciliated cells, These microvilli-like cilia are expressed on the apical surface of both cell lines but they seemed to be more numerous on IB3-1 cells, as they were in monocultures (figure 4.14) Cell morphology of both cell lines is of a typical epithelial appearance, and individual cells appear to be closely connected to neighbouring cells forming a sheet like blanket (on top of the HPF, since these cells are not visible). Scale bar: C38 = 20 µm; IB3-1 = 10 µm n=2)

Both co-culture model systems showed the same appearance of epithelial cells, which form a confluent cell layer with close contacts to neighbouring cells that seem to adjoin closely to their neighbours, in the "tight" physiology typical of the airways epithelium. The expression of microvilli seems to be more numerous on HPF-IB3-1 compared to HPF-C38 co-culture, in agreement with the data from the epithelial mono-cultures (figure 4.14), which again indicates that the presence of HPF did not inhibit the epithelial cell differentiation.

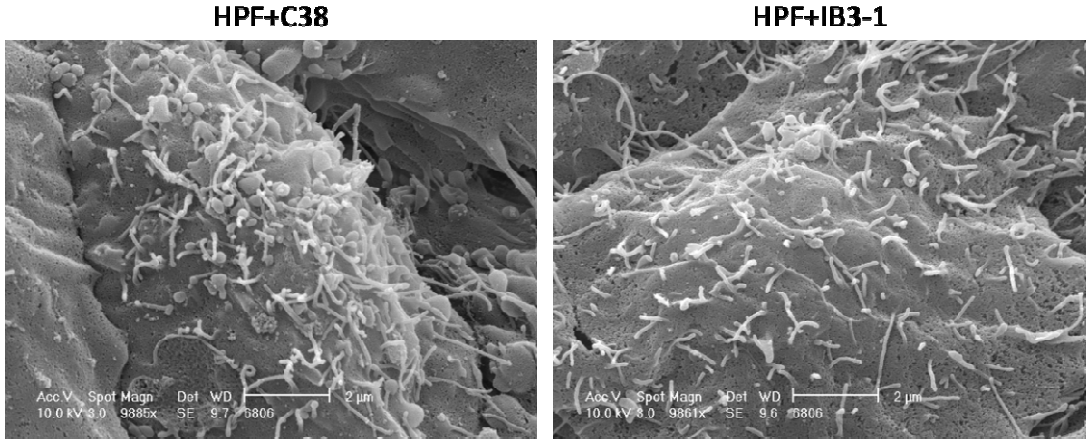

Figure 4.17 Scanning electron micrographs of HPF-C38 and HPF-IB3-1 co-cultures showing cellular microvilli like extensions on the apical membrane.
Co-cultures of HPF-C38 and HPF-IB3-1 showed a heterogenous population of apical extensions, short and thick microvilli and some longer immature cilia. Scale bars in both pictures are 2 µm.

HPF-C38 and HPF-IB3-1 (figure 4.17) were also looked at in more detail and the micrographs revealed that in both co-cultures the microvilli appear to be more cilia-like, as they appeared to be longer and thinner in appearance but were still relatively unorganised and scattered over the whole apical surface of these epithelial cells.

The third epithelial cell line that was used to establish mono- and co-culture models was Calu-3. These were also analysed for the epithelial cell appearance and were viewed under SEM to reveal more detail of cell morphology and epithelial specific features.

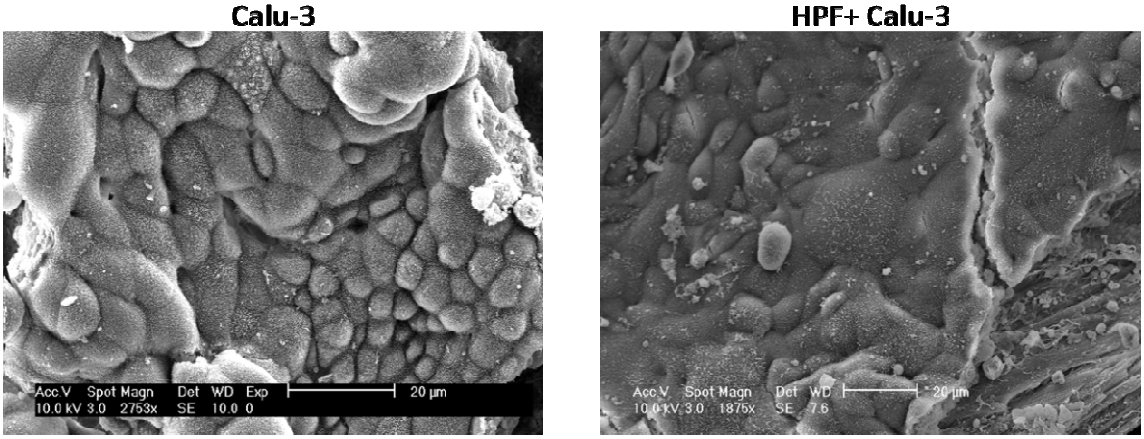

Figure 4.18 Scanning electron micrographs of Calu-3 mono-culture (left picture) and HPF-Calu-3 co-culture (right picture).
For mono- and co-cultures of Calu-3 a confluent tight epithelial cell layer was observed. All cells are in contact with their neighbouring cells in both models, which appeared seamless for adjacent cells. Scale bars in both pictures are 20 µm. In the right bottom corner of HPF-Calu-3 co-culture picture the confluent HPF cell layer was revealed as the epithelium has been damaged during processing. These images indicate that HPF do support epithelial cell growth at ALI when grown in co-culture.

The scanning electron micrographs showed that the epithelial cell layer in mono- and co-culture form a confluent layer of Calu-3 cells with the typical cobblestone morphology (figure 4.18)

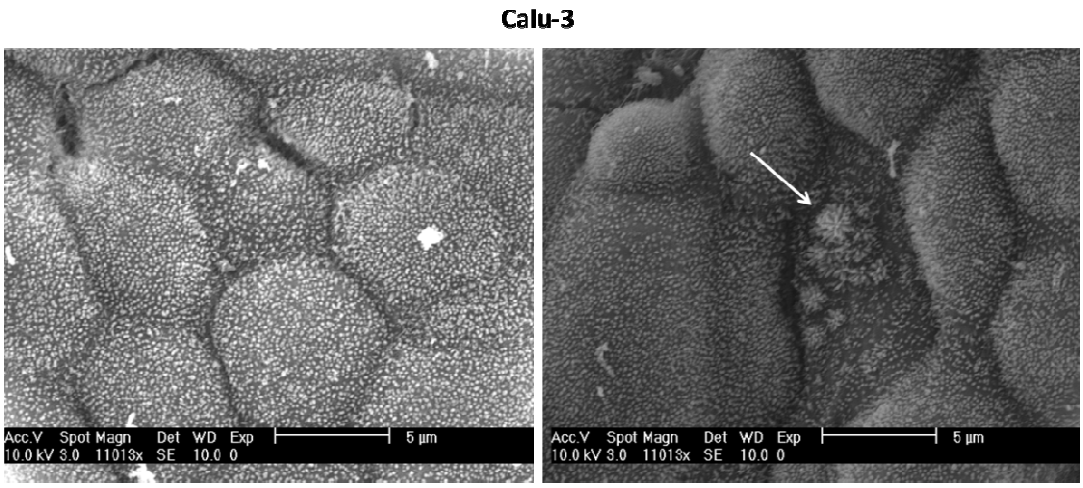

Figure 4.19 Higher magnification of Scanning electron micrographs of a confluent Calu-3 mono-culture showing that these cells were covered in microvilli extensions as well as some longer more organized immature cilia (arrow on right image) on the apical surface of these epithelial cells. Again all cells are in contact with their neighbouring cells as typical for epithelial cells. Scale bars in both pictures are 5 µm.

Calu-3 mono-cultures were shown to be covered in apical microvilli extensions on most cells. Some of these seemed to be a bit longer and thinner than others especially in areas where two cells meet each other. On a few cells there were bundles of organised looking cilia (figure 4.19, right picture).

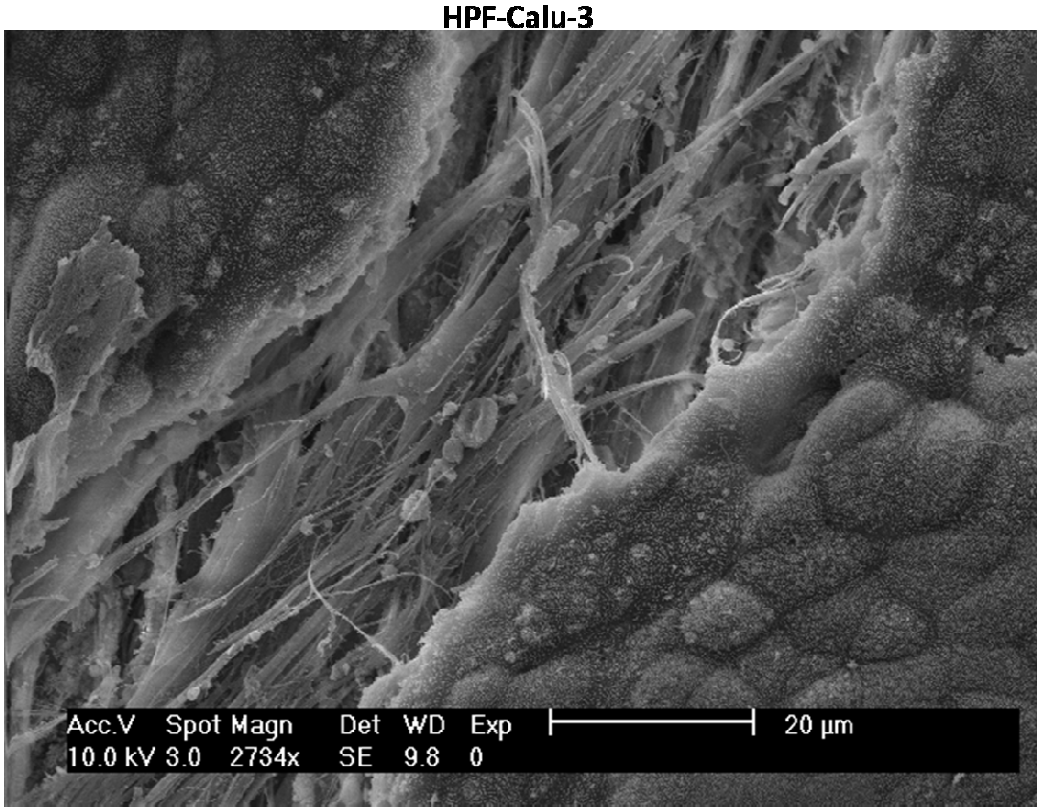

Figure 4.20 Scanning electron micrograph of HPF-Calu-3 co-culture
This picture clearly showed that the epithelial cells form a confluent layer on top of a confluent layer of HPF, which did not inhibit this formation of a tight epithelium, where all neighbouring cells are in contact. Scale bar 20 µm.

The micrograph shown in figure 4.19 shows where the epithelial layer was damaged during processing and this showed very clearly that the HPF layer of cells is present underneath the epithelium without changing its morphological appearance. This image also demonstrates that both cell types look identical in morphology in co-culture and to mono-cultures of HPF and Calu-3 (figure 4.13 and 4.18 respectively).

4.4.4 Transmission electron microscopy of Calu-3 mono and co-culture grown at ALI

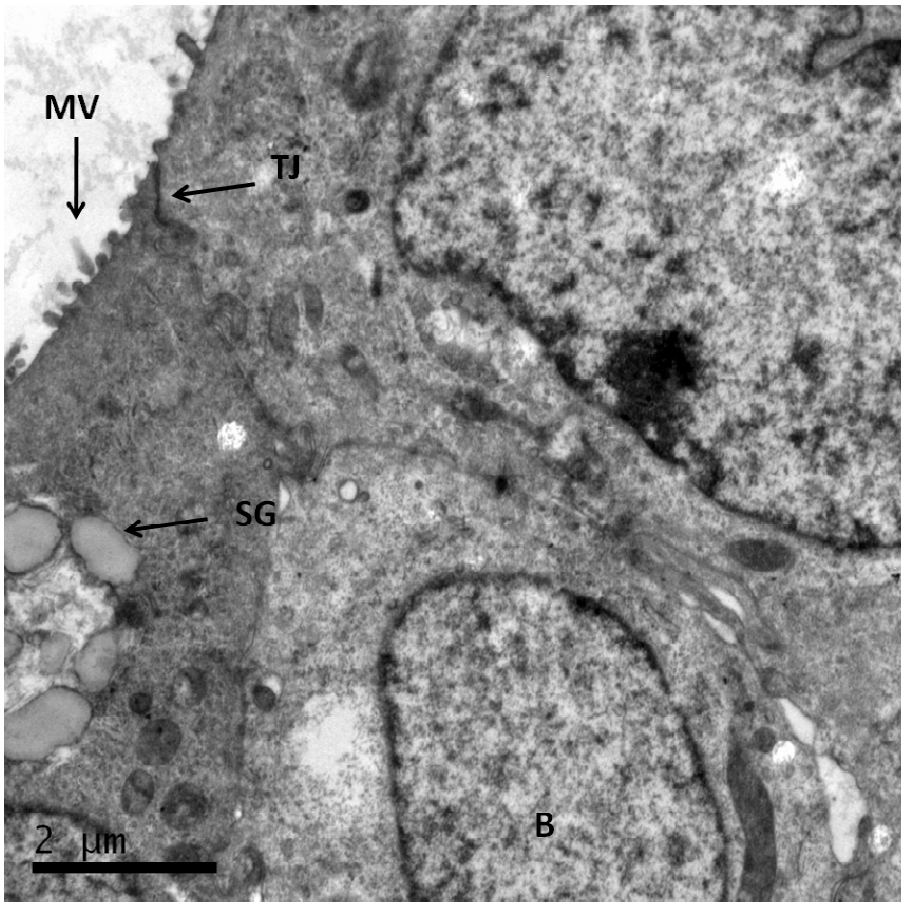

Figure 4.21 TEM micrograph of confluent Calu-3 mono-cell culture at ALI
In this image typical features of pseudostratified epithelium were observed. At the bottom there is a basal (B) epithelial cell that does not reach the "luminal side" and straight above the basal cell, two ciliated cells meet and are connected by a tight junction (TJ) at the most apical point of both cells. Microvilli (MV) expression was observed on these two cells on the apical side of the cells and some secretory granules (SG) were identified on the left side of the image. (n=1)

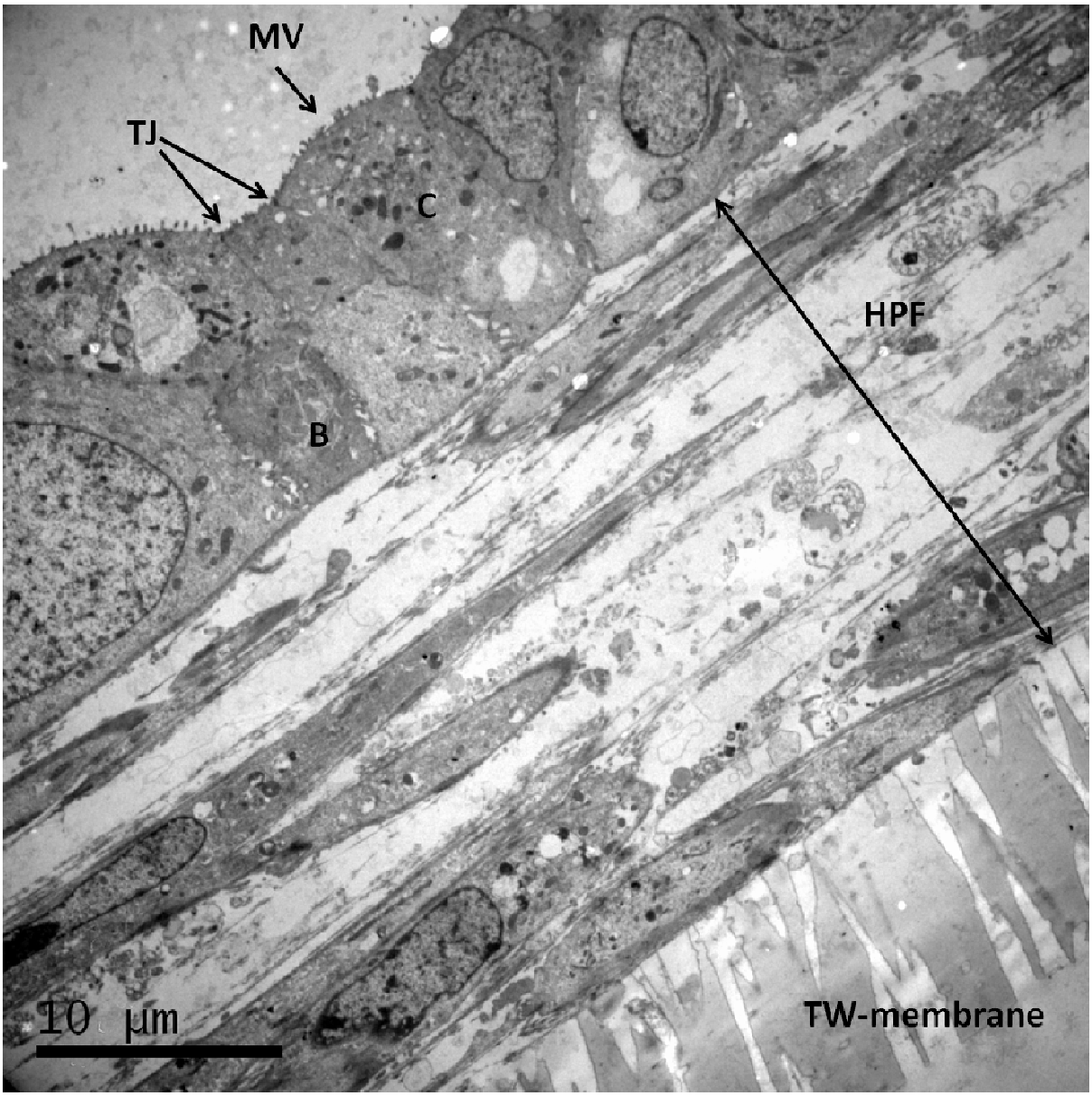

Figure 4.22 TEM micrograph of HPF-Calu-3 co-culture at ALI
In this image of HPF-Calu-3 co-culture typical features of pseudostratified epithelium were observed even when HPF were present and HPF showed a cell multilayer that is clearly distinguishable from the pseudostratified epithelium on top of them. At the bottom of Calu-3 there were several basal (B) epithelial cells that did not reach the "luminal side" but ciliated cells (C) expressing microvilli (MV) were present throughout the apical surface of this cross section with intercellular tight junctions (TJ).

TEM micrographs of Calu-3 mono-culture (figure 4.21) and HPF-Calu-3 co-culture (figure 4.22) showed that these cells established into a pseudostratified epithelium and in both cases the Calu-3 cell layer looks identical and consists mainly of basal (B) and ciliated cells (C) with microvilli (MV). In co-cultures, the HPF cell layer (figure 4.22) is clearly distinguishable from the epithelium as they showed typical morphological appearance being spindle shaped with oval and elongated nuclei. The images indicate that there is a clear distinction between fibroblasts and epithelial cell layer. Fibroblasts are clearly located subepithelial on the TWs and epithelial cells

located on top of the fibroblasts on the luminal side. Furthermore the expression of microvilli was identified for both cultures as well as presence of basal cells. In mono- and co- cultures intercellular tight junctions (TJ) were apparent, along with secretory granules (SG) (figure 4.21 and 4.22).

4.5 Discussion

CF is a multiorgan disease but one organ is affected the most and failure of the airways causes most morbidity in patients. CF lung tissue is not readily available and therefore lots of different models have been developed to enable researching the pathogenesis of CF. Another challenge is to understand the direct link between CFTR mutations and development of lung disease.

Several murine models, of which some are reviewed in Mall (2008), as well as other animal models, such as pigs and ferrets, have been reported (Rogers *et al*., 2008, Sun *et al*., 2010, Mall, 2008) and been used to investigate the pathogenesis of this disease as much as possible but all models have their inherent limitations. This is also true for *in vitro* models of human airway cells, either primary or generated cell lines. Numerous *in vitro* single cell culture models of airway epithelial cells, which are either polarized or non-polarized, have been used in this research field. Single cell culture models of airway cells, especially cultured under submerged conditions have been shown neither to reflect the *in vivo* cell appearance nor to reflect typical cell behaviour in terms of inflammatory responses, for example. Primary human epithelial cells in culture are arguably the closest model for optimal study but in terms of CF, not much is known about the phenotype, which is isolated from patients and how the cells behaviour might have changed over the years facing chronic infection, for example.

4.5.1 There is a need for new cell culture models for CF

The establishment of multicellular co-culture models employing human cells and research on multicell systems are another important step forward to mimic the *in vivo* situations more closely and accurately *in vitro*. These models will be a valuable addition to what has already been established, plus these will enable to look at human intercellular communications, for example fibroblast-epithelial cell communication in response to infection.

It is important for *in vitro* cell culture models that the *in vivo* biological properties of the target tissue, in this case of the bronchial epithelium, are mirrored by the cells used *in vitro*. After determining the basic culture conditions for the mono- and co-culture models (chapter 3), further characterisation of these mono- and co-cultures grown under these defined conditions was necessary.

4.5.2 Cell specific markers for model characterisation

Immunocytochemistry was one method proposed for use to distinguish HPF from the epithelial cell lines in the models, as cell type specific marker antibodies are available to visualise these cells. Optimum concentrations for 1B10 and vimentin were determined before these antibodies were used to stain HPF, C38, IB3-1 and Calu-3 in mono- and co-cultures. HPF and all three epithelial cell lines were also with anti-CK5 and anti-CK8 under submerged conditions as well as mono-cultures at ALI. CKs were first identified in the late seventies and were extensively catalogued by Moll *et al.* in 1982; this catalogue was updated in 2006 (Schweizer *et al*., 2006, Moll *et al*., 1982). Cytokeratins are epithelial specific intermediate filaments that allow researchers to discriminate between tissue specific epithelial cells but also allow them to distinguish between the various stages of differentiation with respect to their CK specific pattern (Purkis *et al*., 1990). For this reason antibodies have been developed against these filaments and the two antibodies used for this project were CK5 and CK8. CK5 is a marker for basal epithelial cells, whereas CK8 is strongly expressed in differentiated simple epithelial cells and has also be found in the respiratory tract, where it is mainly found in the lumen lining cells (Moll *et al*., 2008, Purkis *et al*., 1990).

Submerged cells grow into a mono-layer of cells, which resulted in clear images with low or no background staining (figures 4.1 – 4.4). This was found to be different, when staining was performed on mono-cultures grown at ALI. The high number of cells, which were in multi layers, lead to strong overlapping staining, which made it sometimes difficult to identify single nuclei and get a clear picture of the stained cell markers. This often produced high overall fluorescence staining as well as sometimes overlapping fluorescence signals of green and blue, so it was almost impossible to find the right exposure time to get clear and focused pictures. Another point was the difficulty of getting a reasonable sized area of view that was in focus all around, was also encouraged by the fact that once a TW membrane was excised from the outer support it needed to be handled with extensive care to not start rolling up or to become wavy and distorted.

The TW membrane itself has been reported to be a problem in imaging. This has been shown for Calu-3 mono-cultures before, when grown at ALI and it was also mentioned that staining of cells on TW membranes can appear pixellated, depending on how close to the membrane the images are taken (Grainger *et al*., 2006). This was the same problem when cells were co-cultured at ALI but as shown in this chapter valuable images for analysis can be produced using these methods. Clear quality allow images allow morphology analysis as well as cell type identification.

Analysing these images revealed that 1B10 did not only staining HPF, which was a strong and clean staining pattern for fibroblast surface antigen, but it also showed some staining on the epithelial cells in submerged cultures, which was even stronger at ALI (figure 4.1-4.8). The staining pattern is very different when comparing HPF's staining pattern to the epithelial ones but 1B10 would not be the perfect antibody for identifying HPF in co-cultures.

In contrast both anti-CK antibodies were epithelial cell specific and did not show any reaction with HPF (figure 4.1 and 4.5). Surprisingly CK5 did not stain C38 and IB3-1 under submerged conditions and only very little when grown at ALI, which needed such a high exposure time to visualize that the background staining was almost the same. However, CK5 is strongly expressed in Calu-3, which is paired with CK14 *in vivo* and they are the major CKs in basal cell populations (Purkis *et al*., 1990), which indicates that a basal cell population is present in these cultures, alongside with ciliated cells, which has also been demonstrated in figure 4.21 and figure 4.22. CK8 however, is strongly expressed in all three epithelial cells and the anti-CK8 antibody does not show any reactivity towards HPF.

4.5.3 Cytokeratin differences observed in cultured cell lines

Interestingly the expression pattern of the CK8/CK18 intermediate filaments seems to differ in each cell line. In C38 it looks like the CK8 filaments are polarised to one end of the cell in most cases and only a thin bundle of them appear rather than all around in the cytoplasm (figure 4.2). IB3-1's CK8 expression looks quite different as it seems the positive staining appears in all the spaces between the stained nuclei, which would imply CK8 is present in most areas of the cytoplasm. It looks as if they express more of it and therefore it seems to be denser compared to C38 (figure 4.3). For other CKs it has been shown that the expression pattern does change in response to stimulation. CK13, for example, has been reported to be increased by exposure to chronic inflammation, which is the characteristic condition of the CF airways. When chronic inflammation is present in CF airways the epithelium undergoes squamous metaplasia and/or repair after tissue injury (Mithal and Emery, 1976, Sajjan *et al*., 2000b). CK8 expression in CF has been addressed in one report concerning the localization of CFTR in homozygous Δ-F508 cell lines. This work showed that reorganization of CK18 within the CK8/CK18 network after treatment with curcumin was accompanied by relocalization of CFTR to the plasma membrane in CF cells, whereas curcumin had no effect on non-CF cells (Calu-3) (Lipecka *et al*., 2006), indicating that there is a difference in CK expression present comparing non-CF and CF cells.

C38 has been transfected and stably expresses CFTR, this could be accompanied by CK8 changes in terms of reorganization of the intermediate filaments to enable the translocation of CFTR to the plasma membrane and as shown here (figure 4.6, 4.7 and 4.9) the CK8 expression pattern differs comparing C38 and IB3-1, which could be shown to exactly this change in intermediate filament organisation.

Transfection, but mainly transformations, can have an influence on CK expression as discussed later on in this chapter. CK8 staining of Calu-3 cells is also different in its appearance compared to the other two epithelial cell lines, which is possibly down to their different origins as Calu-3 derived from a bronchial adenocarcinoma whereas IB3-1 are from a CF patient. This could mean that there are fundamental differences in their morphology and maybe even some functional (apart from being immortal) characteristics. Interestingly it has been reported that although CK5/CK14 expression levels are similar in tumours compared to normal epithelial cells, low or no CK5 is expressed in adenocarcinomas (Moll *et al*., 2008). This was not the case here for CK5 epxression of Calu-3, where strong CK5 expression was apparent(figure 4.4 and 4.8) and which has been described before (Daniel and Burnett, 1991). On top of these findings Calu-3 have been reported to have an abnormal set of chromosomes, which could affect several cell function and expression patterns but this has not been linked directly to CK expression until now (Shan *et al*., 2011).

For all epithelial mono-cultures at ALI their CK staining patterns were different compared to submerged cultures, which is plausible as the epithelial cells differentiate and so the cytoskeleton needs to rearrange and establish cell-cell junctions such as desmosomes for example, which involves intermediate filaments (Green and Jones, 1996). As just mentioned CKs play a role in cell-cell junctions, for example as well as providing stability. Therefore these inherent structures and their distribution will change when a single cell is integrated into tissue formation (Moll *et al*., 2008). C38 staining with CK8 looks like the filaments are more dispersed at ALI, rather than in big bundles and were found at the very top level of cells, the apical layer. Filaments stretched out through the whole cytoplasma and were now even found even stretching over the nucleus (figure 4.6). CK8 staining of IB3-1 at ALI looked now very similar to C38's staining pattern at ALI just slightly less diffuse, which supports the finding that no visual morphologic differences are observed comparing non-CF and CF cells (Yankaskas *et al*., 1985).

Cytokeratins were thought to be inert structures for mechanical support but nowadays we know that they are active, mobile and have also been reported to be polarised in the case of simple

epithelial cells, which are themselves polarised with an apical and basolateral side (Oriolo *et al*., 2007). The cytokeratin expression profiles of epithelial cells can change for several reasons. One is naturally occurring in terms of embryogenesis, where keratin 5 expression increases in certain simple epithelial cells (epidermis) as these become basal cells for the development of a stratified epithelium (Coulombe *et al*., 1989). Another reason for dramatic changes in the expression profile is being cultured *in vitro*. For example, it was reported that rat tracheobronchial epithelial cells changed cytokeratin expression over short period of 72 h when first cultured *in vitro*. The expression of CK14, a basal cell marker and partner of CK5, significantly increased over this time and it was also shown that prominent morphological changes took place (Yamamoto *et al*., 1998).

As C38 and IB3-1 were isolated in 1992 and have been cultured for a long time *in vitro*, it is impossible to tell whether this weak CK5 staining of C38 and IB3-1 is correlated to these cells being cultured and no CK staining data of the first days of *in vitro* culture is available for comparison.

Changes in CK expression has been reported to occur when cells are transformed with the oncogenic SV-40 virus, which has been described as "a stage specific process in which normal patterns of differentiation are progressively altered over time following infection" (Steinberg and Defendi, 1985). The infection with the virus created human epidermal keratinocytes with different variations of keratin expressions, some of which were deficient for some CKs of their normal keratin cytoskeleton, including CK5, which correlates with negative staining found in C38 and IB3-1. These changes happened after a short time (10-15 passages) after the infection. These changes were ongoing for months after the infection and led to the loss of certain keratins but also to the appearance of some others, which were not present in uninfected cells (Steinberg and Defendi, 1985). In second report of keratins expressed in human keratinocytes, it has been shown that there is an overall reduction in keratin RNA synthesis and that Ck5 is markedly reduced in transformed cells compared to their parental strain, which usually expressed a stable pattern. For these keratinocytes it has been observed that viral transformation changes the differentiation ability (Morris *et al*., 1985). The human bronchial epithelial cell line VA 10, which was used for investigating branching morphogenesis in 3D culture were reported to maintain their basal cell appearance in terms of CK14/17 expression even though they partially differentiated into a type II alveolar cell (Franzdottir *et al*., 2010). This shows that CK expression, like it was found in the presented models, does not have to change completely in terms of switching on and off certain CKs in correlation with differentiation.

Furthermore pseudostratified epithelium as it is found in the respiratory tract is, generally speaking, classed as a simple epithelium, which has been shown to have a fairly simple cytokeratin profile as well. Usually these epithelial express CK8/CK18 and sometimes CK19 and CK7. The stratified epithelial cells express a more complex range of CKs as they express 1-6 and 9-17, which is thought to be linked to their stratification (Bosch *et al*., 1988). The pseudostratified epithelium has all cells attached to the underlying BM but as its name implies, the stratification is not complete. It has been reported that in some cases typical CKs of stratified epithelia (CK5) can be co-expressed with those of simple epithelia (CK8) (Bosch *et al*., 1988), which is exactly what was observed in HPF-Calu-3 co-cultures. The fact that CK5 is not expressed in C38 and IB3-1 does therefore not rule out the presence of a basal cell population in these culture models, since CK8 was present.

For the purpose of identifying epithelial cells in the co-culture models, CK8 is a reliable epithelial cell marker that is expressed by all three epithelial cell lines in submerged culture conditions as well as in ALI conditions. Also this CK was not found to be expressed by HPF and therefore would be of use in defining the location of each cell type within the co-culture. Immunocytochemistry staining was also performed on all three co-culture models grown at ALI and they were stained with 1B10, CK5 and CK8 the result observed was 1B10 showing a strong staining in HPF-C38 and HPF-IB3-1, which showed that HPF are present in the model and no mesenchymal to epithelial transition has taken place. In HPF-Calu-3 co-cultures staining seems to be weak for 1B10 but it seems like the epithelial cell layer is very tight in this and either did not let the antibody penetrate through to the HPF or if the antibody got to its antigen, the fluorescence is not coming through and could not clearly be captured. CK5 expression again is low in HPF-C38 and HPF-IB3-1 but strong in Calu-3, whereas CK8 is strongly expressed in all three co-cultures (figure 4.9). CK8 staining for HPF-C38 looked different to mono-culture staining, as in the way that the filaments are now located all around the nucleus outlining the cells morphology. In the top left corner there is only DAPI stained HPF nuclei and it seems that some C38 have been lost during processing. IB3-1 and Calu-3 in co-culture show the same staining patterns as in mono-culture at ALI.

4.5.4 A closer look at mono- and co-cultures

As immunocytochemistry used on excised TWs and imaging these cultures using fluorescence microscopy is not the ideal method for identifying two different cell types in one model system when looking at them from the apical side, another method needed to be applied. Cross

sectioning of these models seemed the next best way of reaching this aim. As there is no histology department and not the essential equipment at Aston University these experiments as well as the electron microscopy analysis were performed in Birmingham University. Handling TW membranes is challenging and often the membrane is lost on the way through processing (figure 4.11 and 4.12).

H&E staining of HPF, C38 and IB3-1 mono-culture cross sections (figure 4.11) were obtained and show a tall pseudostratified like epithelium. In the next set of images antibodies to vimentin were used as an HPF marker to localize the fibroblasts in the co-culture system of C38 and IB3-1 (figure 4.12) and fibroblasts were shown to be located underneath the epithelium, which maintained a pseudostratified like appearance. No fibroblast overgrowth was seen in any of these co-cultures, despite previous reports of fibroblast over-proliferation, especially when isolating primary epithelial cell from tissue samples (Heckman, 1983). The same finding was observed for HPF-Calu-3 co-culture when analysed using SEM (figure 4.18 and 4.20) as well as TEM (4.20 and 4.21). In all these micrographs the two layers of different cell types are readily to distinguish; with HPF underneath showing their specific morphology as being spindle shaped and Calu-3 are located on top of these presenting a tight confluent pseudostratified epithelium. The confluent layer showed the typical cobblestone like appearance. When looking at the SEM micrographs it seems like in the co-culture the apical surface of Calu-3 is a little smoother (figure 4.18). TEM further revealed that in both models Calu-3 showed the appearance of a pseudostratified epithelium with TJs, microvilli and secretory granules. These pictures are very similar to what has been shown by Grainger in 2006 and supports our theory that fibroblasts do not overgrow the epithelium under the right conditions (Grainger *et al*., 2006).

C38 and IB3-1 mono- and co-cultures were also analysed using SEM and morphologically there is no difference to be seen comparing mono-cultures to co-cultures and the typical cobblestone appearance is very prominent. Both cell lines grew to a confluent epithelium in both model systems. It was also possible to verify the presence of apical microvilli and cilia like extensions, which in some cases could be immature cilia, on all seven model systems by using SEM (figure 4.15, 4.17 and 4.19). For Calu-3 mono-cultures it has been summarised before that there can be significant differences in certain cell specific features such as cilia when comparing results of different laboratories, which could be down to different culture conditions and passage numbers (Grainger *et al*., 2006). Grainger also found small microvilli on submerged cell cultures, which were more prominent in cultures at ALI but this work also emphasizes that they resemble immature cilia or extended microvilli rather than fully developed ones (Grainger *et al*., 2006).

To conclude, the methods presented in this chapter and their outcomes helped to successfully identify cell type specific marker antibodies for future analyses of cross-sections. Anti-vimentin and anti-CK8 can be used for identifying HPF and epithelial cells, respectively in the co-culture models. IHC staining on co-cultures and vimentin staining on cross sections also show that there is no fibroblastic overgrowth, fibroblasts are located subepithelial and both cell types show specific morphology, next to expressing cell type specific markers.

5 Chapter 5 Characterisation of functional properties of mono- and co-culture models

5.1 Introduction

The data presented in the previous chapters have determined that collagen type IV is a suitable growth substrate, have identified the culture media to be used for co-culturing HPF together with one of the epithelial cell lines C38, IB3-1 or Calu-3 and have used antibodies against cell type specific markers in paraffin embedded cross sections to identify the distribution of each cell type in the co-culture models. Furthermore the presence of microvilli and immature cilia was verified in cultures at air-liquid interface and now the next task was to look at additional essential features for modelling human airways *in vitro* for non-CF and CF human airways. Further analysis of mono-and co-cultures was carried out to identify whether additional essential features, such as epithelial cell integrity and barrier formation (tight junctions) were present in this model. Furthermore, preliminary analysis of air-surface secretions (ASL) for the presence of mucin and proteolytic enzyme activity was carried out.

The airway epithelium is a complex and active physicochemical barrier and primarily protects the airways and the whole organism by acting as a first line of contact with the outer environment. It protects the respiratory tract from inhaled particles and pathogens, such as bacteria and viruses. The cells in the respiratory tract form complex connections with adjacent cells to ensure three dimensional structure and normal function is maintained (Breeze and Wheeldon, 1977, Gail and Lenfant, 1983). In the large airways a pseudostratified epithelium is found, where every epithelial cell is attached to the basement membrane (BM). The major epithelial cell types of the large airways are basal, columnar, ciliated and secretory cells (goblet cells) (Knowles and Boucher, 2002, Breeze and Wheeldon, 1977). In order to maintain the integrity of the epithelium, the basal cells are strongly attached to the BM through hemidesmosomes, which additionally give mechanical integrity. In a similar manner basal cells are connected to the columnar cells via desmosomes, which also create trans-cellular networks throughout the tissue and provide certain resistance to mechanical stress (Green and Jones, 1996). Additionally epithelial cells form a permeability barrier between two compartments, which inhibits molecules and ions crossing via the intercellular route (Stevenson *et al*., 1986). This sealing connection where the columnar cells are adjoined is achieved by the tight junctions (TJ), which are located most apically and close to the lumen. This strong barrier formation plays its important role in lung innate immune function by selective blocking of the paracellular pathway (Farquhar and

Palade, 1963). Another part of innate immune protection in the airways is the production and secretion of mucus. Secretory epithelial cells produce mucus that forms a thin lining on the luminal side of the airways on top of the PCL (Gail and Lenfant, 1983, Knowles and Boucher, 2002). The viscoelastic mucus layer is responsible for entrapping any inhaled pathogens and inhaled particles, which reach the airways through every breath (Voynow and Rubin, 2009). Airway mucus consists of a mixture of water, ions and mainly of two of the oligomeric glycoprotein mucins, MUC5AC and MUC5B (Thornton and Sheehan, 2004, Breeze and Wheeldon, 1977). This mixture of mucus, entrapped pathogens and particles is in healthy airways moved towards the pharynx by the net movement of cilliary beating, where it is swallowed (Gail and Lenfant, 1983).This defence mechanism is called the mucociliary escalator (MCC) (Crystal *et al*., 2008). As already described in chapter one this whole situation is different in CF, where a CFTR mutation leads to dehydrated ASL and hypersecretion of mucus, which then entraps and impairs cilliary beating leading to vast amounts of stationary mucus in the lungs that invites bacteria to colonize this habitat and results in chronic infections in CF patients (Knowles and Boucher, 2002, Matsui *et al*., 2005).

Normal lung function is controlled by various different mechanisms, cells and chemokines, just to name a few influential aspects. The structure of the lung, which is supported by the scaffolding network of ECM is also essential to maintain lung function and the ECM synthesis and degradation needs to be tightly controlled. Next to other residential cell (neutrophils), epithelial cells secrete proteolytic enzymes called matrix metalloproteinases (MMPs), which are responsible for controlled ECM turnover (O'Connor and FitzGerald, 1994). Human bronchial epithelial cells express MMPs, which have a broad spectrum of degradation substrates, including collagen IV and, importantly MMPs were found to be increased in bronchoalveolar lavage (BAL) of CF children compared to healthy controls (Sagel *et al*., 2005). The increased amount of MMPs has been suggested to play a role in the tremendous tissue destruction found in some CF patients but further research is need to link certain MMPs directly to CF lung disease (Sagel *et al*., 2005, Greenlee *et al*., 2007).

5.2 Aims

Since the growth and culturing conditions have been determined and cell specific marker antibodies that can be used to distinguish between epithelial cells and HPF it was now the aim to characterise the typical, essential functional features for these *in vitro* models.

In order to achieve further characterisation the electrical resistance properties of HPF and the epithelial cell lines were determined in mono- and co-cultures.

The second aim was to investigate the expression and location of the TJ protein ZO-1 under submerged condition and at ALI.

Another important feature that was analysed was the directional MUC5AC secretion of mono- and co-cultures.

The last aim here was to investigate whether there is any MMP-activity present in apical secretions of these cultures.

5.3 Methods

5.3.1 Cell culture on TWs

Briefly, (see 2.4.6 for detailed description) for mono-cultures of HPF, C38, IB3-1 and Calu-3, cells were seeded apically onto TWs at 3 x10^4 cells/well. Calu-3 cells were seeded in full DMEM/F12, whilst C38 and IB3-1 cells were seeded in AEM. HPF cells were seeded in HPFM. Cells were cultured submerged for four days before ALI was established on epithelial cell cultures. The apical medium on HPF was refreshed as was the basolateral medium of all cells. Medium was refreshed twice a week.

For co-cultures HPF cells were seeded exactly as described above and apical HPFM was removed 4 days after seeding to allow epithelial cell seeding in AEM at a cell density of 5 x 10^4 cells/well. At this point also the basolateral medium was changed to the epithelial medium used in the co-culture. Again four days of submerged culture followed before ALI was established to induce differentiation of the epithelial cell layer.

5.3.2 Transepithelial electrical resistance (TER) measurements on mono- and co-cultures

TER is a measure of the resistance to ion flux across the epithelial cell layer, which reflects the degree of confluence and the tightness of the cell barrier (Foster *et al*., 2000). TER is commonly used in airway related research working with cell cultures at ALI as it is convenient, reliable and non-destructive to the cell layer. It allows monitoring of the growth of epithelial tissue cultures *in vitro* and to monitor the formation of epithelial cell layer integrity, which also indicates the presence of TJs.

Briefly, the chopstick electrodes were sterilised by immersing in 70% ethanol for approximately 15 min before being used as shown and described in detail in chapter 2.9. The electrodes were rinsed every time in ethanol before moving on to the next cell line or another multi-well plate. Three readings per TW were taken and background TER of a collagen IV coated TW was subtracted from the average and multiplied by the surface area of the culture to give TER readings as $\Omega \times cm^2$. TER was measured on day 0, day 4, day 7, day 11 and day 14 to create a TER profile for each epithelial cell line and for HPF

5.3.3 Dot blot analysis of MUC5AC

Air surface liquid secretions (from the apical compartment) of mono- and co-cultures, as well as basolateral medium, were collected on day 0, 10 and 14 to be analysed for the presence of MUC5AC, the main mucin in airways mucus. Sample collection as described in 2.10 was either followed by immediate analysis or samples were frozen at -80°C till analysis. After preparing the dot blotter, samples were applied and driven through the nitrocellulose membrane by a vacuum pump. Blocking of the membrane was followed by exposure to a monoclonal anti-mouse muc5AC antibody overnight before the membrane was treated with secondary antibody followed by ECL for 5 min to make the visualization of MUC5AC possible by detection of the exposed light on a photographic film.

5.3.4 Zymography for MMP activity

Gelatin zymography is a common method for analysing the expression of the gelatinase matrix metalloproteinase-2 (MMP-2) in cells and media samples. After collecting the ASL samples (carried out in the same manner as for the dot blot) these were either frozen at -80°C or directly prepared for zymography. The 10% gelatin zymograms were bought from Invitrogen (Novex®) and were used as recommended by the manufacturer.

After the gels were mounted in the running chamber and Tris-glycine SDS running buffer was added the samples were loaded in non-reducing sample buffer (Novex®) as well as recombinant MMP-2 standard (Peprotech; 62kDa).

After electrophoresis at 120V for 60 minutes, the gel was washed (4 x 15 min) with 2.5% (v/v) Triton X-100 in incubation buffer (50mM Tris-HCl, 10mM $CaCl_2$, 1mM $ZnCl_2$, pH 7.6), to remove SDS and renature the MMP-2 species in the gels. Gels were then placed into incubation buffer for at least 72 hours at 37°C. This induced gelatin lysis by renatured MMP-2. The gels were incubated in staining solution (1 % (w/v) Coomassie Blue G in 40 % (v/v) methanol and 10 % (v/v) acetic acid) for 20 minutes, before de-staining in 40 % (v/v) methanol and 10 % (v/v) acetic acid until the gelatinolytic activity was apparent as clear bands on a blue background.

5.4 Results

5.4.1 TER profiles of mono- and co-cultures grown at ALI

The development of co-culture systems (HPF-C38, HPF-IB3-1 and HPF-Calu-3) was the overall main aim of this study, as the first step towards developing a useful test platform system to mimic CF airways disease. In order to validate the disease model, it was also just as important to produce a reliable, realistic non-CF model of the airways. One critical feature of such a model is epithelial cell integrity and associated barrier function (Foster *et al*., 2000). The TER reflects this integrity, although the intrinsic electrical resistance across the epithelial cell layer is different for the different epithelial cell lines used for the co-culture models.

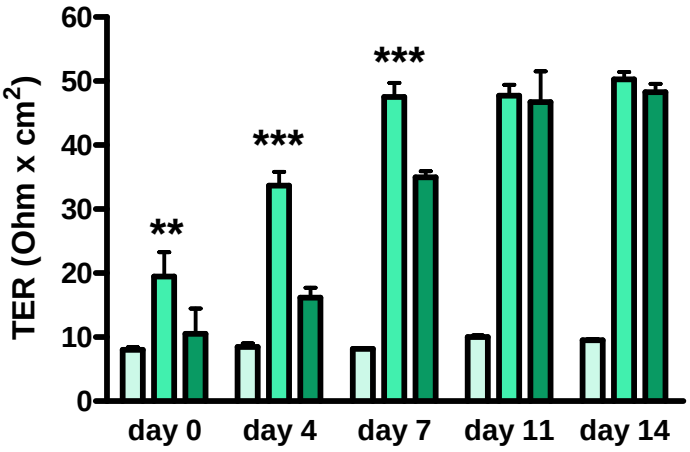

Figure 5.1 TER development of monocultures of HPF (pale green), C38 (light green) and co-cultures of HPF-C38 (dark green) over 14 days.
HPF were kept submerged and C38 mono- and co-cultures were cultured at ALI. Mono-and co- cultures with C38 show equal values of TER after 11 days of culture. TER is expressed as measured resistance per area (cm^2) of the cell layer and background TER of an empty TW coated with collagen IV was subtracted from the reported values. In each of the three separate experiments TER of 8 individual TWs were measured in triplicate and data are presented as mean ± SD.

In figure 5.1 changes in TER over time for HPF-monoculture (submerged), C38 mono-culture and HPF-C38 co-culture (both at ALI) are presented. HPF mono-cultures do not show any increase in the electrical resistance measured over this 14 day period shown and the overall average TER was 8.85 ± 0.89 Ω x cm^2. In contrast, the TER of the epithelial cell line C38, even in mono-culture, at 19.48 ± 3.8 Ω × cm^2, was more than twice as high compared to HPF mono-culture on day 0. For C38, TER showed a steady increase up to day 7, after which it plateaued. The final TER of C38

mono-culture after 14 days at ALI was 50.3 ± 1.12 Ω × cm^2. HPF-C38 co-culture TER was observed and found to show the same steady increase as mono-cultures but the initial TER on day 0 was with 10.52 ± 3.94 Ω x cm^2 significantly lower than for C38 in mono-culture. Throughout the increase over time this significantly lower TER was also found for day 4 and day 7 but by day 11 the co-culture TER reached 48.26 ± 1.32 Ω x cm^2, which is equivalent to C38 mono-cultures.

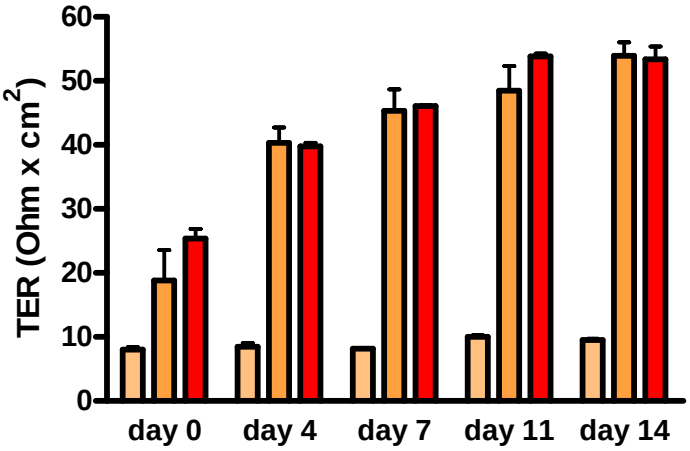

Figure 5.2 TER developments of HPF (pale orange), IB3-1 (orange) and HPF-IB3-1 (red) over 14 days. HPF were kept submerged and IB3-1 mono- and co-culture were cultured at ALI. Mono-and co- culture show equal values from day 4 onwards up to day 14 of culture. TER is expressed as measured resistance per area (cm^2) of the cell layer and background TER of an empty TW coated with collagen IV was subtracted from the reported values. Data presented as mean ± SD. In each of the three separate experiments 8 TWs were measured in triplicate.

In figure 5.2 TER profiles for HPF mono-cultures, IB3-1 mono-culture and HPF-IB3-1 co-culture are presented. The electrical resistance of HPF mono-cultures did not increase over time. IB3-1 mono-cultures show a steady increase from day 0 (18.81 ± 4.78 Ω × cm^2) up to day 14 (53.92 ± 2.1 Ω × cm^2). The TER of HPF-IB3-1 co-cultures showed a rapid increase straight after day 0 (25.38 ± 1.5 Ω × cm^2) and carried on increasing till day 11 (53.83 ± 0.45 Ω × cm^2). On day 14 TER was found to be at the same level (53.38 ± 1.99 Ω × cm^2) for mono- and co-cultures.

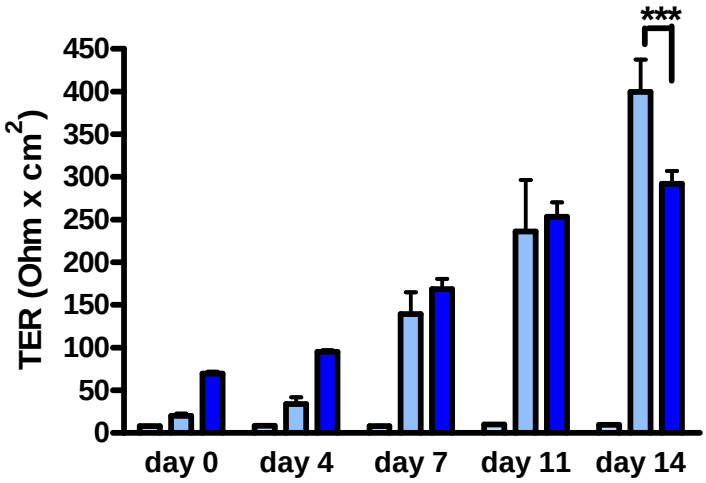

Figure 5.3 TER profiles of HPF (pale blue), Calu-3 (light blue) and HPF-Calu-3 (dark blue) over 14 days. HPF were kept submerged and Calu-3 mono- and co-culture were cultured at ALI. Mono-and co- culture show equal values on day 11 but at day 14, the mono-culture continued to increase and reached a significantly higher TER compared to the co-culture. TER is expressed as measured resistance per area (cm^2) of the cell layer and background TER of an empty TW coated with collagen IV was subtracted from the reported values. Data presented as mean ± SD. In each of the three separate experiments 8 TWs were measured in triplicate.

HPF mono-cultures do not show an increase in electrical resistance over time, whereas TER measurements of Calu-3 mono-cultures and HPF-Calu-3 co-cultures showed a steep, time-depdendent increase in TER. TER of the mono-culture model was measured and was 19.99 ± 2.54 $\Omega \times cm^2$ on day 0. The electrical resistance continously increased up to 399.55 ± 37.99 $\Omega \times cm^2$ on day 14. The co-culture model had an electrical resistance of 69.66 ± 2.14 $\Omega \times cm^2$ on day 0 and after a steady increase over 14 days the resistance reached 291.96 ± 14.90 $\Omega \times cm^2$. The TER of HPF-Calu-3 co-cultures was significantly lower on the last day measurment compared to the mono-culture.

5.4.2 Immunostaining of zonula occludens-1 (ZO-1) on mono- and co-cultures

5.4.2.1 ZO-1 expression under submerged conditions

Immunostaining with an antibody against the tight junction protein ZO-1 was performed on submerged mono-cultures of HPF, C38, IB3-1 and Calu-3 on collagen IV coated 4-well slides.

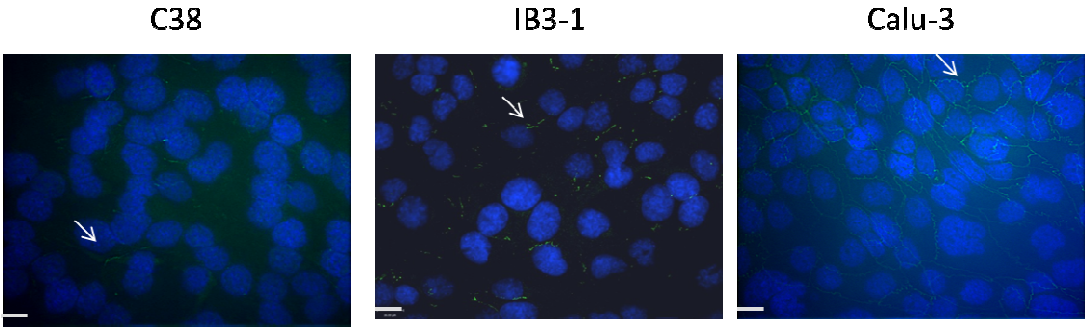

Figure 5.4 Representative images for staining of the tight junction protein Zonula Occludens-1 (ZO-1) in submerged mono-cultures of C38, IB3-1 and Calu-3 cultured on collagen coated 4-well slides. Nuclei were counterstained with DAPI and ZO-1 is shown in green (FITC labelled antibody). Magnification is 63 times and the bar corresponds to 31µm. The images show that ZO-1 is present in epithelial cells cultured under submerged conditions (arrows). Images are representative of 3 individual experiments, with duplicate wells stained per antibody.

For submerged C38 and IB3-1 mono-cultures low expression of the TJ protein was observed and looked rather scattered throughout the cell, rather than concentrated at the intercellular junctions. C38 seemed to show less expression compared to IB3-1. Calu-3, however, strongly expressed ZO-1 and the protein showed the typical junctional staining pattern for distribution can be identified as continous rings at cell-cell contacts.

5.4.2.2 ZO-1 expression in mono-and co-cultures grown at ALI

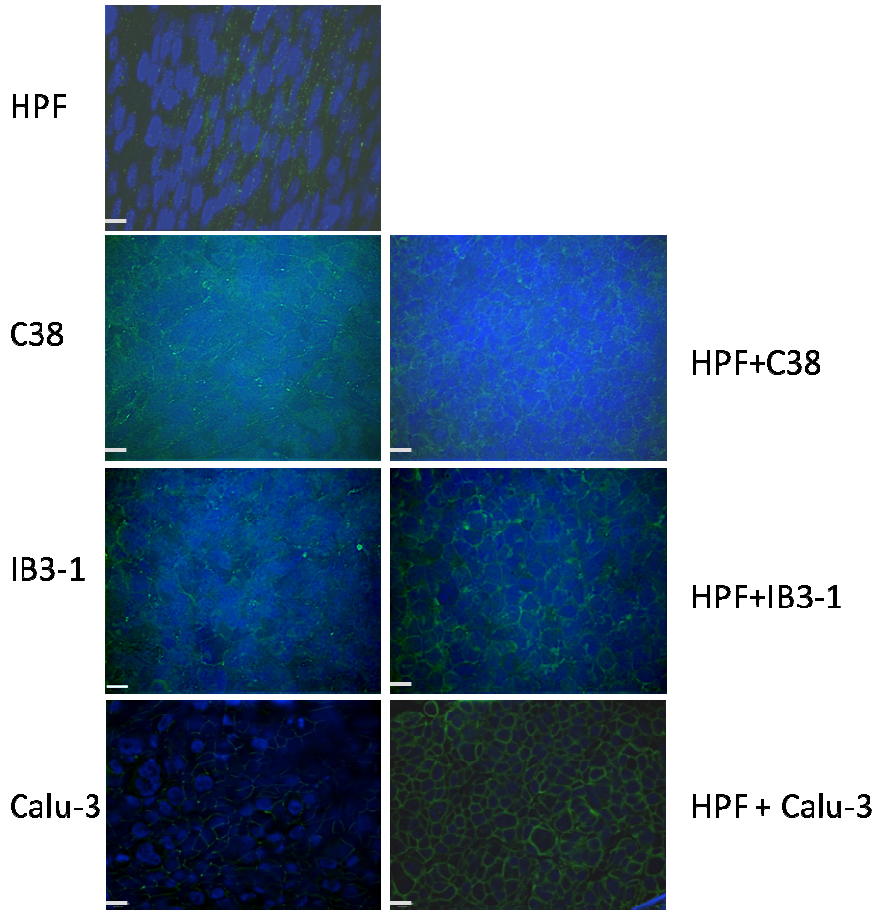

Figure 5.5 Representative immunostaining images for the tight junction protein Zonula Occludens-1 (ZO-1) of mono- and co-cultures of HPF, C38, IB3-1 and Calu-3 cultured on TW for 14 days at ALI but HPF were kept submerged throughout the experiment. Nuclei were counterstained with DAPI and ZO-1 is shown in green. All epithelial cells showed ZO-1 expression in mono-and co-cultures when grown at ALI. HPF subepithelial cell layers promote TJ protein expression in co-culture. Magnification is 63 times and the bar corresponds to 31µm. Images are representative of 3 individual experiments, with duplicate wells stained per antibody.

When HPF were cultured on TWs under submerged conditions they showed scattered staining for ZO-1 (green), whereas the epithelial cell lines all show strong, peripheral ZO-1 expression in mono-and co-culture. Mono-cultures of C38 and IB3-1 both show the typical ZO-1 staining, which is shown as the ring staining around each cell with partial interruptions where scattered green staining is shown. Calu-3, in contrast, show a very clear staining only showing typical ring staining of the tight junction protein ZO-1 around the cell periphery with no background or cytoplasmic staining. The co-culture staining profiles of ZO-1 for HPF-C38 and HPF-IB3-1 look very similar to the patterns observed for the mono-cultures, with the major difference that staining now appeared more concentrated at the periphery. ZO-1 staining is very prominent and again the typical ring staining around cells was observed. For HPF-Calu-3 the staining is very

prominent, clean and showed what looked like a nice even layer of cells, which were all in focus and so were the typical ZO-1 rings around each cell. Z-stack analysis of the ZO-1 staining revealed that this was at the most apical, luminal aspect of each cell type, which correlates with the location of ZO-1 in the TJ complex.

5.4.3 MUC5AC secretion by mono-and co-cultures

Identification of mucin secretion was performed using dot blot analysis with a monoclonal antibody against MUC5AC, which is one of the two major mucins expressed in the airways (Guyonnet Duperat *et al*., 1995, Kirkham *et al*., 2002). Samples from apical and basolateral compartments of all mono- and co-cultures were analysed (figures 5.6 and 5.7).

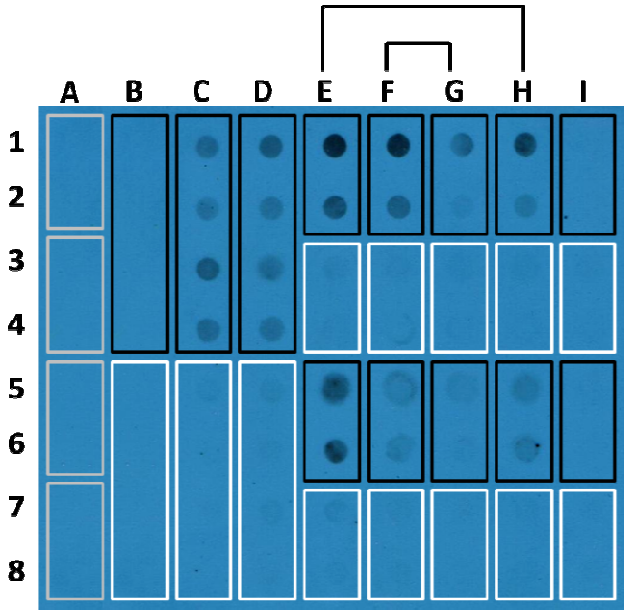

Figure 5.6 Dot Blot analysis of mucus secretion by HPF (submerged cultures), C38 and IB3-1 in mono-and co-culture before establishing ALI and at ALI by using a MUC5AC antibody.
Negative controls and samples were applied in duplicates as followed: lane A: 1+2 = PBS; 3+4 = 1% BSA, 5+6 = AEM, 7+8 = DMEM/F12; For all the other lanes, black boxes indicate where apical samples were loaded and white boxes show where basolateral samples were loaded. All samples were loaded as the neat, undiluted sample (upper dot of the pair) and 1:2 dilution (lower dot). In lane B samples from submerged HPF cultures are shown: 1+2, apical, day 10, 3+4, apical, day 14, 5+6, basal, day 10, 7+8, basal, day 14; In lane C the results of HPF-C38 co-culture are shown: 1+2, apical, day 10, 3+4, apical day 14, 5+6, basal, day 10, 7+8, basal, day 14; In lane D the results for HPF-IB3-1 are shown in the same order as just described for HPF-C38. In lanes E and H the results for IB3-1 mono-cultures are shown with day 10 (1-4) and day 14 (5-8), where 1+2 were apical, 3+4 were basal and where 5+6 were apical and 7+8 were basal again. In lanes F and G the results for C38 mono-cultures are shown with day 10 (1-4) and day 14 (5-8). 1+2 in both lanes were apical samples and 3+4 were basal samples; also in both lanes 5+6 were apical samples and 7+8 were basal samples. In lane I samples of C38 (1-4) and IB3-1 (5-8) mono-cultures before ALI was established, were analysed with 1+2: C38, apical and 3+4: C38, basal; 5+6, IB3-1, apical and 7+8, IB3-1, basal. Overall MUC5AC was only observed in apical samples and only after ALI was established. All controls were negative for MUC5AC. Data presented are representative of 3 individual experiments.

The dot blot shown in figure 5.6 shows the results of qualitative analysis of MUC5AC secretion by the established mono- and co-culture models. HPF in submerged culture did not secrete MUC5AC either to the apical side (lane B, 1-4) or to the basal side (lane B: 5-6). C38 mono-cultures (lane F and G) and IB3-1 mono- cultures (lane E and H) were found to secrete MUC5AC to the apical surface (black boxes), no MUC5AC was observed in the basolateral medium (white boxes). However, this was only observed after ALI was established as apical and basolateral samples of day 0 (before ALI was established) were analysed and were negative for MUC5AC for C38 mono-culture (lane I, 1-4) and also for IB3-1 mono-culture (lane I, 5-6). HPF-C38 co-culture (lane C) only secreted MUC5AC to the apical side (black box) and again the basolateral medium (white box) was found to be negative for MUC5AC antibody staining. For HPF-IB3-1 co-culture (lane D) the same secretion profile was observed. MUC5AC was observed in the apical samples (black boxes) but not in the basolateral medium (white box). The controls, including full culture medium, shown in separate grey boxes in lane A (each in duplicate) were all found to be negative for MUC5AC.

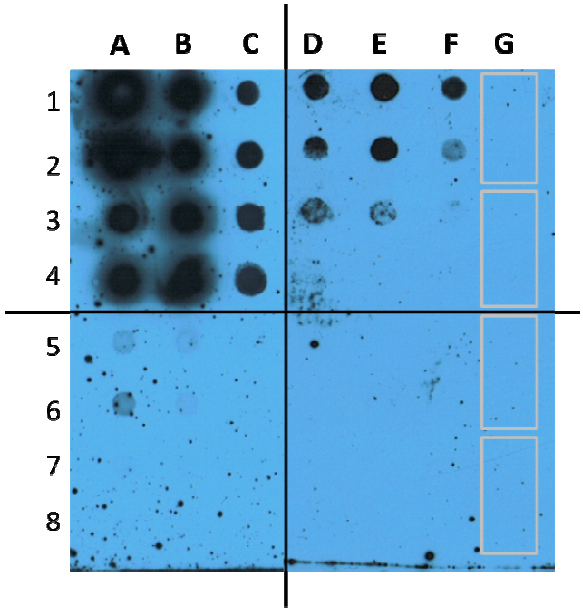

Figure 5.7 Dot blot analysis of mucus secretion by Calu-3 mono- and co-culture using a MUC5AC antibody
In lanes A and B samples of Calu-3 mono-cultures were applied with apical samples in well 1-4 and basolateral medium samples in well 5-8 in the dilutions mentioned in 2.10. Lane A shows samples collected on day 10 of culture, whereas lane B shows samples of day 14 of culture. In lane C samples of day 0 (before ALI) are shown. Using the same dilutions HPF-Calu-3 co-culture samples were applied in the same way, with apical samples in wells 1-4 of lane D-F and with basolateral samples in wells 5-8 of these lanes. Here lane D shows the results for samples collected on day 10 and lane E shows samples of day 14 of culture. Samples collected on day 0 were loaded in wells of lane F. The last lane shows 4 grey boxes where negative control were applied in duplicates in the following order: PBS (1+2), 1% BSA (3+4), AEM (5+6) and DMEM/F12 (7+8). Data presented are representative of 3 individual experiments.

The dot blot presented in figure 5.7 shows the results of MUC5AC analysis for secretions from Calu-3 mono-culture as well as for HPF-Calu-3 co-culture. Mono-cultures of Calu-3 secreted MUC5AC into the apical compartment only, when cultured at ALI (A and B, 1-4). MUC5AC was also detected in apical supernatants that were collected on day 0, which was before ALI was established (C, 1-4). For none of these serial dilutions (well 1-4) a difference in detection signal was observed. The same secretion pattern was observed for HPF-Calu-3 co-cultures but the MUC5AC detection signal was quite weak and even was undetectable in any of the highest dilutions (lane D-E, well 4). Basolateral medium samples were observed to be negative for all analysed samples apart from one (lane A, 6), which showed weak staining. No MUC5AC was detected in any of the controls applied to the dot blot (G).

5.4.4 MMP activity in apical surface liquid

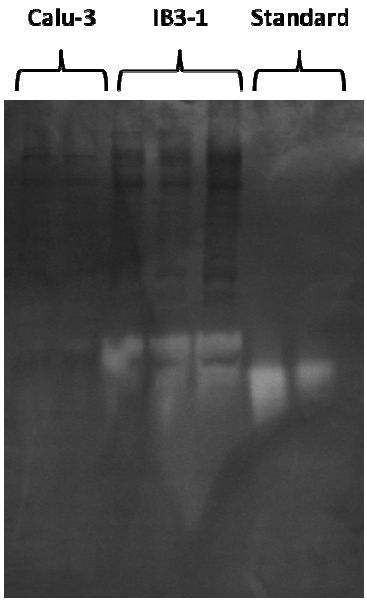

Figure 5.8 MMP activity in apical supernatants of Calu-3 and IB3-1
Aliquots of apical secretions of Calu-3 and IB3-1, along with purified, activated MMP-2 were applied to a zymogram. Activated MMP-2 exhibits a distinct characteristic band of active enzyme with a molecular weight of 62 kDa (as given by manufacturer). Apical secretions of Calu-3 did not exhibit any bands, whereas the apical secretions of IB3-1 mono-culture contained two distinct bands, which were presumably the latent form of the enzyme at an apparent molecular weight of 72 kDa, higher than the activated form of enzyme with a molecular weight of 62kDa. The lower band falls exactly in the same position as the purified MMP-2. (Gel is representative of n=3).

Figure 5.8 shows air surface liquid collected from Calu-3 mono-cultures and IB3-1 mono-cultures that were subjected to gelatin zymography. Only for IB3-1 mono-culture's apical secretions proteolytic activity was observed as clear bands on the zymogram, where the substrate gelatin

was digested. In those three lanes of IB3-1 secretions, two distinct bands were identified, of which the lower one corresponds to activated MMP-2. The second band was seen at a slightly higher molecular weight, corresponding to the inactive form of the enzyme at 72kDa. Thus these data show that these enzymes are proteases of metalloproteinase origin. As there are two bands shown for IB3-1 secretions this indicates presence of the active as well as the latent form of a gelatinase, which is most likely MMP-2.

5.5 Discussion

In this chapter the main focus was directed towards essential airway features which make the proposed *in vitro* models a valuable tool for further investigating CF airways pathogenesis in addition to other *in vitro* and *in vivo* models that already exist.

The co-culture models employed fibroblasts as well as epithelial cells in one system with direct intercellular contact. The challenge was now to demonstrate that these fibroblasts support epithelial cell differentiation, which has partially been done in chapter 3 and 4 but additional essential features were analysed here. One of these features was cell layer integrity of the epithelial cell layer and whether this is supported by the addition of pulmonary fibroblasts. This was addressed by TER measurements as well as staining for TJ protein expression (ZO-1). Furthermore, directional MUC5AC expression by mono-and co-cultures was observed as well as proteolytic enzyme activity in apical secretions of these models.

5.5.1 Epithelial cell layer integrity in mono- and co-cultures of C38 and IB3-1

One way of analysing epithelial barrier formation was to measure the electrical resistance across the epithelial cell layer in mono-and co-cultures. TER is an accepted way of measuring cell layer integrity, and is convenient, reliable and non-destructive. Measurements were first carried out on mono-cultures of HPF, C38, IB3-1 and Calu-3. For HPF no change in electrical resistance across the TWs over time could be identified, which fits with results found by Myerburg *et al*. (2007) and supports that HPF do not form impermeable barriers themselves (day 0 = 8.03 ± 0.42 Ω x cm^2, day 14 = 9.54 ± 0.18 Ω x cm^2), even when grown over 14 days to a multilayered cell population on TWs. This indicates that the TER measured in these co-culture models is established by the epithelial cell layer and is not a function of the fibroblasts.

Similar final TER values were reached, when comparing mono-cultures of C38 and IB3-1; thus after 14 days at ALI C38 mono-culture TER was 50.29 ± 1.12 Ω x cm^2, whereas for IB3-1 mono-culture it was 53.93 ± 2.11 Ω x cm^2. IB3-1 TER was higher but was not found to be significant, which was different after the addition of HPF.

At first the TER results of co-cultures of HPF with C38 or IB3-1 were similar. HPF-C38 co-cultures reached a final TER of 48.26 ± 1.32 Ω x cm^2 and IB3-1 in co-culture with HPF reached a final TER of 53.38 ± 1.99 Ω x cm^2. Interestingly when cultured with HPF, IB3-1 reach significantly higher TER values for every day of measurement compared to HPF-C38 co-cultures. These findings match what has already been reported by Nilsson *et al*. (2010), who also found higher TER in CF

cell lines compared to non-CF cell (Nilsson *et al*., 2010) This provides evidence for a relevant feature of the disease in the presented model systems. As suggested by Nilsson *et al*. (2009) functional CFTR is necessary for correct ionic conductance of TJ. This group has compared the cell lines CFBE410- (CF) and its plasmid corrected counterpart CFBE41o- pCep4 for their ability to establish an epithelial barrier over time on TWs and found that the CF cells used here also show a significantly higher TER. Furthermore this group has also shown that the CFTR inhibitor $CFTR_{inh}$-172 is able to increase the TER in the non-CF cell line 16HBE14o-, also shown in the reverse direction by using CFTR activators, which caused a drop in TER in 16HBE14o indicating a connection between CFTR and TJ ionic permeability. On top of these findings this group has shown that the decrease in TER was paralleled by an increase in permeability and the other way around (Nilsson *et al*., 2010). Similar results were found when HTE cells were treated with CFTR inhibitor. The short circuit current measured before and after complete abolishment of CFTR function was shown to drop (Perez *et al*., 2007).

The data of this present study show clearly that HPF do not interfere with epithelial differentiation at ALI in these co-culture models under the determined culture conditions and in support of others the co-cultures show the CF presented higher TER.

5.5.2 Epithelial cell layer integrity of Calu-3 mono- and co-cultures

Looking at TER of Calu-3 mono-cultures and co-cultures it was clear that after the culture period of 14 days at ALI the co-culture TER is still lower (291.96 ± 14.9 $\Omega \times cm^2$) compared to the mono-culture (399.55 ± 37.99 $\Omega \times cm^2$). For Calu-3 mono-cultures it was reported that in general the maximum TER is reached between day 10 and 14 of culture (Florea *et al*., 2003), however this is the first time that a co-culture of this combination and under these conditions was analysed and it is possible that these cultures take longer to reach similar values to the mono-culture. These were not monitored for longer time periods in this project but there is a possibility that the co-culture will eventually reach a TER as high as measured for the mono-culture. Calu-3 mono-cultures were shown before to vary in the final TER values drastically. There is a report showing TER >1000 $\Omega \times cm^2$ (Mathia *et al*., 2002) compared to some that show TER values around 300 $\Omega \times cm^2$ (Grainger *et al*., 2006, Wan *et al*., 2000), even when using similar techniques. These reports underline that there is a lot of variability found in final TERs for this cell line, which can be due to laboratory differences in handling cells or different passage numbers, for example, as well as growth conditions, such as choice of medium, choice of TW, as they are available with different membranes, as well as choice of TW coating (fibronectin, collagen IV etc.).

The data presented here (figure 5.3) show that from day 7 onwards Calu-3 mono and co-cultures both show a higher TER than the highest TER measured for C38 or IB3-1. Again Calu-3 do show essential features for airway modelling *in vitro,* such as TJ protein expression (Wan *et al*., 2000) and MUC5AC secretion, however tremendous differences of TER values between mono- and co-cultures seen here and the fact that Calu-3 vary in their cell layer integrity drastically among different reports, it seems that Calu-3 are not the cell line of choice to serve as a non-CF model. Additionally this cell line was shown to also behave very differently compared to the other two epithelial cell lines and therefore Calu-3 are not likely to be a non-CF control in this project. As Calu-3 originated from a lung adenocarcinoma lines there are likely to be tremendous differences between these cells and C38 and IB3-1. It is recommended to only compare cells that originate from the same donor as the genetic background will be different otherwise, which might have an effect on several things rather than only CFTR function (Nilsson *et al*., 2010, Perez *et al*., 2007). Also if cells originate from different diseases it is likely that they are exposed to very different environments, which will influence the cell phenotype.

5.5.3 Tight junction expression in mono-and co-cultures

In addition to the TER data presented in this study fluorescence microscopic imaging of ZO-1 protein expression was performed following immunostaining of submerged mono-cultures as well as mono- and co-cultures grown at ALI. ZO-1 protein is one of the structural components of TJs and was identified in 1986 to be present in epithelial cells (Stevenson *et al*., 1986). Out of the three junctions forming the apical complex, TJs are located most apically and serve to selectively regulate the passage of neutral molecules and ions through the paracellular space. ZO-1 is found in the region of apicolateral borders, where cell-cell contacts have been arranged (Stevenson *et al*., 1986) , thus its expression pattern is typically rings around every cell. ZO-1 was clearly expressed in all three epithelial cell lines as shown in figures 5.4 and 5.5. For C38 and IB3-1 typical, ring ZO-1 staining could only be observed when grown at ALI in mono-and co-cultures. In these cultures TJs appeared nearly continuous rings that were localized to the periphery of each cell.

The ZO-1 expression observed for submerged C38 and IB3-1 was less continuous with greater incidences of breaks compared to the cultures at ALI, which indicates that tight junctions are formed under certain conditions by these two cell lines. In submerged culture, cells show a different cell organisation and morphology compared to their pseudostratified like appearance at ALI and this could prevent the ZO-1 colocalization with other necessary TJ components to form the typical ring seen around each cell at ALI. It appears that these cells might need the

establishment of an ALI, by exposure to air on the apical side of the epithelium, to promote differentiation into a pseudostratified confluent and tight epithelium as it has been reported before, for primary nasal and bronchial epithelial cells (Wiszniewski *et al*., 2006).

In contrast, Calu-3 show clearly defined typical ZO-1 expression around each cell under submerged conditions as well as at ALI (figures 5.4 and 5.5). It has been previously reported that Calu-3 cultured on TW show continuous "rings" of stained ZO-1 around each cell under both conditions but in that study staining was weaker at ALI. This is in contrast to what was observed here but it could be down to the fact that cultures presented here were grown on 4-well slides rather than on TWs for submerged conditions (Grainger *et al*., 2006).

Taken together results from TER measurements and ZO-1 staining both support that epithelial cell layer integrity is established and it was observed that in C38 and IB3-1, which had less continuous ZO-1 staining the TER was also lower when compared to Calu-3, which is in accordance with ZO-1 staining, which is continuous in Calu-3. Even though cell layer integrity is measured by TER and TJ are responsible for ion flow across the paracellular pathway in epithelial cells there is no clear, direct correlation between TER and ZO-1 expression and the data should be interpreted with care (Claude and Goodenough, 1973). For Calu-3 it has been shown that cells cultured at ALI produce a cell layer with greater similarity to the airway epithelial morphology and electrical resistance *in vitro* (Grainger *et al*., 2006), which is presented here for C38 and IB3-1. It is clearly shown that in terms of TER and ZO-1 expression, C38 and IB3-1 are able to establish a cell layer showing airway epithelial cell morphology.

Another interesting finding in the present study was that HPF grown under submerged conditions on TWs showed positive staining for ZO-1, which was perhaps not expected, given the absence of TER in this cell type, however, there were no continuous strands of ZO-1 at all, only very scattered staining with no particular organisation. This shows that the expression of ZO-1 protein does not automatically mean functional TJ are present at site of adjacent cells. And this also underlines that the expression of ZO-1 protein does not necessarily correlate with increasing TER.

The presence of TJs has been noted in other fibroblasts, for example, fibroblasts of ocular tissue were found to show similar pattern of TJ protein expression as is shown here. For ocular fibroblasts it was reported that these tight junctions did not form a belt and rather looked like TJ fragments scattered on the plasma membrane (Raviola *et al*., 1987).

5.5.4 MUC5AC secretion of mono- and co-cultures

Another desirable essential feature for *in vitro* airway model systems would be measurable mucus production by the epithelial cells. As described in chapter 1 air-surface liquid (ASL) is the collective name for mucus and the periciliary liquid layer (PCL) underneath the mucus. In comparison to mucus, the PCL has low viscosity and lubricates the cilia, which can then beat frequently to propel the overlying mucus towards the pharynx (Thornton and Sheehan, 2004, Livraghi and Randell, 2007, Gail and Lenfant, 1983). One defining feature of CF airways pathogenesis is the impaired mucociliary escalator and deficient mucus clearance, as a result of dehydrated mucus and changes in the amount of gel-forming mucins expressed in the airways. It has been reported that MUC5AC mRNA was found upregulated after stimulation with bacterial products, however, mRNA might not be an accurate indicator of mucin quantities in the mucus-gel as these glycoproteins can be expressed and be stored in cells before secretion (Kirkham *et al*., 2002). Furthermore the quantitative analysis of mucins found in different airways diseases (asthma, CF and COPD) showed that MUC5AC and MUC5B glycoforms appear in variable amounts but also that MUC5B in its low charge glycoform is increased in CF and COPD compared to asthma (Kirkham *et al*., 2002, Thornton and Sheehan, 2004). MUC5B needs to be analysed in future experiments to verify it is secreted and to see whther it is hypersecreted in IB3-1. In the study presented here all epithelial cell lines used were shown to secrete MUC5AC apically and C38 and IB3-1 only secrete MUC5AC after ALI was established. This identification was carried out by dot blot analysis, which does not allow accurate quantitation of mucin and therefore whether IB3-1 hypersecrete mucin still needs to be addressed.

Dot blot analysis of Calu-3's apical secretions showed very strong signals and they therefore appeared to secrete relatively high levels of MUC5AC, which could be down to the cellular origin, as they are derived from serous cells of submucosal glands of human airways (Ramesh Babu *et al*., 2004).

HPF mono-culture were screened in this dot blot to see whether they still show a typical non-mucus producing phenotype and also to confirm that the mucus produced could be correlated to the presence of differentiated epithelial cells in the co-culture models. IB3-1 and C38 continued to apically secrete MUC5AC at ALI in mono- and co-cultures. The fact that co-cultures show MUC5AC secretion indicates that HPF do not inhibit this epithelial cell feature, which has also been found for HPF-Calu-3 but in this co-culture model MUC5AC secretion seemed to be much lower compared to the mono-cultures. Interestingly C38 and IB3-1 do not show any sign of MUC5AC secretion before ALI, indicating that differentiation is a necessary event before mucin is produced or released by these cells.

All models presented here produce and secrete MUC5AC, apart from the HPF mono-culture, which underlines the epithelial origin of MUC5AC found in these apical secretions. C38 and IB3-1 only secreted MUC5AC after ALI was established and again this is completely different to Calu-3 mono- and co-culture models, which secrete mucus before the established ALI.

5.5.5 MMP activity in apical secretions of mono- and co-cultures

Another hall mark of CF is the progressive lung destruction, which eventually leads to death, but for which the underlying mechanisms have not yet been completely elucidated. Proteolytic enzymes, such as MMPs, were reported to be upregulated in CF for the first time in 1995 (Delacourt *et al*., 1995). These enzymes are collectively able to degrade most ECM components and therefore some of them (MMP-8, MMP-9) have been suggested to be partially responsible for this lung destruction if excessively secreted and activated. In BAL fluid of patients, even with mild lung disease, MMP-8 was found to be increased by 300-fold and MMP-9 by 116-fold. In the same study it was also shown that, although the specific MMP inhibitor, TIMP-1, was increased, this was only by 6-fold, which leads to an imbalance in activation and inhibition of the MMPs. Therefore there is evidence that these enzymes play a role in the persistent lung destruction in CF (Ratjen *et al*., 2002).Whilst there is limited evidence on the roles and expression of MMP-2 in the CF lung it has been suggested that MMP-2 is involved in CFTR channel regulation in human airways. Using a MMP inhibitor (phenanthroline) an increase of the short circuit current was observed. Additionally antibodies against MMP-2 were shown to have the same effect, whereas recombinant MMP-2 reduced the current. These short circuit profiles match the ones of CFTR (Duszyk *et al*., 1999) and further investigation of MMP-2 in CF airways might elucidate what direct input this enzyme has.

Other MMPs have also been reported to play a role in CF lung disease. MMP-9, which is mainly produced by neutrophils has been found to be increased in sputum of children with CF and was correlated to increased inflammation and decreased lung function (Sagel *et al*., 2005), for example.

In the presented models, proteolytic activity in apical secretions was only observed for IB3-1 mono-cultures, which was shown as clear bands on the zymogram (figure 5.8), where the substrate gelatin was digested. In each sample of IB3-1 two clear bands were observed, with the second band located slightly higher on the zymogram, indicating a higher molecular weight, corresponding to the inactive form of the enzyme, whereas the lower one was located corresponding to the MMP-2 standard used, which had a molecular weight of 62kDa. Thus these data show that these enzymes are proteases of metalloproteinase origin. As there are two bands

shown for IB3-1 secretions this indicates presence of the active as well as the latent form of the present MMP, which is most likely MMP-2. MMP-2 is secreted by structural cells, such as epithelial cells but has not been extensively studied in CF. However, it has been reported that MMP-2 is upregulated by neutrophil elastase and this was found on mRNA level as well as for the enzyme activity in cell supernatants. Latent and active MMP-2 were significantly different compared to control (unstimulated) cells (Geraghty *et al*., 2007). This finding is very interesting as CF is characterised by an intense neutrophil dominated airway inflammation and huge amounts of free elastase have been reported to be present in CF patients, even in early stages of the disease (Konstan *et al*., 1994, Khan *et al*., 1995).

In conclusion the results presented in this chapter show that the presented mono- and co-culture models have additional features, which are required to develop a successful co-culture model for non-CF and CF human airways *in vitro*. For all models employing epithelial cells, it was shown that epithelial cell layer integrity was established after 14 days of culture and that the tight junction protein ZO-1 was also expressed in the apicolateral periphery of epithelial cells. Furthermore it has been shown that MUC5AC is secreted apically by the epithelial cells but not by HPF. Apical secretion were also analysed for proteolytic activity, which was only observed for IB3-1. All together it becomes clear that in mono- and co-cultures using these epithelial cell lines CF features are presented in the CF models but only when compared to their corrected counterpart, not when compared to Calu-3. The models presented show potential for further analysing the disease pathogenesis of CF.

6 Chapter 6 Inflammatory response of mono-and co-cultures to LPS

6.1 Introduction

It is known that CF lungs provide an environment which is readily infected by bacteria and that the developing chronic infection leads to significant lung damage and eventually results in patient mortality (Boucher, 2007).

The airway epithelium is the interface between airways and inhaled pathogens where the epithelium plays a vital role in innate immune responses. The epithelium presents a physico-chemical barrier and once this barrier is penetrated by bacteria, epithelial cells have direct contact with these opportunistic pathogens. Detection of bacterial infections in general occurs via pathogen-associated molecular patterns (PAMPs), such as LPS (Schroeder *et al*., 2002). LPS is an integrated component of the outer membrane of gram-negative bacteria and can be shed by bacteria during growth, exposure to antibiotics and death. These PAMPs are then presented to the host airway epithelial cells as well as to immune cells, which express pattern recognition receptors (PRR) on their cell surface and upon binding trigger a highly pro-inflammatory response. This response involves the production and release of pro-inflammatory cytokines and chemokines such as IL-8, which is considered the most important inflammatory mediator in CF. IL-8 causes a sustained neutrophil influx to the site of infection, where they in turn compound the initial infection and this leads to a non-resolving cycle of inflammation (Akira and Takeda, 2004, Ratjen, 2009).

LPS can mediate its pro-inflammatory effects in part, at least, by binding to the PRR Toll-like receptor 4 (TLR). TLRs play a crucial role in innate immunity as these receptors recognize the invader and rapidly cause an inflammatory response including the secretion of IL-8 (Greene *et al*., 2005b). In this specific case, TLR-4 cannot work on its own but rather a receptor complex delivers the signal. There are three core receptors in this signalling pathway of LPS, which are CD14, TLR-4 and MD-2. CD14 does not have an intracellular domain and cannot deliver a signal across the epithelial membrane and therefore transfers LPS to the complex of TLR-4/MD-2 to ensure downstream signalling occurs.TLR-4 has an intracellular domain that then recruits other proteins involved in this pathway to translocate NF-κB from the cytoplasm to the nucleus, where this complex then can activate the transcription of target genes, such as IL-8 (Kim *et al*., 2005a).

Common pathogens found in CF lungs are the opportunistic bacterium *P. aeruginosa* (Kipnis *et al*., 2006) and *B. cepacia*, which in the last two decades has gained notoriety as a pathogen in CF (Silipo *et al*., 2007) and isolated LPS of these types of bacteria were analysed here for their imunnologic affect on established mono- and co-cultures. Cell culture supernatants were analysed for IL-8 secretion as well as cell layer integrity and cell viability to test the functionality of these models compared to existing literature.

6.2 Aims

In this chapter submerged mono-cultures as well as mono-and co-cultures at ALI were exposed to LPS of *B. cepacia* J2135 and LPS of two different *P. aeruginosa* strains. One was a commercially available LPS from serotype 10 *P. aeruginosa* and the other was *P. aeruginosa* 50DR isolated from CF sputum and provided by Prof Peter Lambert, Aston University. The aim was to investigate the influence of these common immunostimulatory stimuli found in CF on the presented models and to compare results with other published literature to determine functionality of these models and how well they reflect CF.

In order to achieve these aims it was necessary to analyse cell viability of submerged mono-cultures as well as of mono-and co-cultures at ALI after exposure to these three types of LPS.

Furthermore monitoring of the cell layer integrity by measuring TER before and after the challenges was essential to estimate the affect LPS has on the barrier formation of mono-and co-cultures at ALI.

Another important aim was to measure the concentration of the chemokine IL-8 after exposure to LPS to identify whether an inflammatory response is induced or not

6.3 Methods

6.3.1 Cell viability assay – Cell Titer Blue™ (Promega)

Cell Titer Blue (CTB) is an endpoint assay that provides a homogeneous, fluorometric method to monitor cell viability. Viable cells, which are metabolically active, can convert resazurin (blue with little intrinsic fluorescence activity) into its highly fluorescent product, called resorufin (pink). CTB was directly applied to fresh cell medium after samples have been collected and the plate was then incubated for 2 h at 37 °C prior to analysis. The fluorescent intensity was measured on a standard multiwell fluorescent plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with a 560 nm excitation, and 590 nm emission wavelength.

6.3.2 Transepithelial electrical resistance (TER) measurements

TER of mono- and co-cultures on TWs was measured using an Epithelial Voltohmeter with STX2 chopstick electrodes (World Precision Instruments) to monitor confluence and barrier formation of the epithelial cell layer. TER was measured on day 14 or just before exposure to live bacteria to verify epithelial cell confluence and intactness of epithelial cell layer and TJ formation.

TER values reported in this work take into account the area of the cell layer (area of TWs = 0.33cm^2) and are expressed as Ωcm^2. Triplicate measurements were taken for TW and the background resistance, which was typically between 100-120 Ωcm^2 (cell-free collagen IV coated TW) was subtracted from the average of a triplicate measurement.

TER was measured immediately before and straight after the 24 h incubation with live bacteria to analyse whether this cell layer integrity was affected and changed compared to the control TWs.

6.3.3 Bacterial growth conditions

P. aeruginosa 50DR and *B. cepacia* J2315 were grown in MHB at 37°C and placed in a shaker at 150 rpm over night or were plated out on MHA plates and incubated in a 37°C incubator. Bacteria were also stored on MicroBank beads (Pro-Lab Diagnostics, Ceshire, UK) at -70°C until required.

6.3.4 Phenol extraction of LPS

The detailed description of LPS extraction can be found in Chapter 2.13. Briefly LPS was isolated from *Pseudomonas aeruginosa* 50DR (*P. aeruginosa*) and *Burkholderia cenocepacia* J2315 (*B. cenocepacia*), which are both CF relevant bacterial strains. These bacteria cultured overnight on MHA plates at 37 °C. On the next day, one colony was picked to be used to inoculate another overnight culture (100ml) in sterile MHB at 37 °C on a shaker. Afterwards a sterile MHB was inoculated, grown over night and transferred into 2 litres of sterile MHB the next day and incubated 48 h. Test for purity of the bacterial cultures was carried out before each culture was centrifuged to pellet the bacteria. After washing these were re-suspended in sterile water and aqueous phenol (80 °C) was added and the suspensions were stirred for 30 min followed by centrifugation for 20 minutes. Centrifugation yielded 2 distinct phases, the upper phase (containing the LPS) was recovered and transferred into a cellulose acetate dialysis membrane tubing and dialysed against running water for 4 days to remove the phenol. After collection of the dialysate, magnesium sulphate was added to a final concentration of 10 mM to encourage LPS micelle formation. After ultracentrifugation pellet of LPS was re-suspended in 10 ml sterile H_2O and frozen in liquid nitrogen and LPS was freeze dried, weighed and a stock solution prepared and sterilised by filtration. This was further diluted in sterile AEM (serum free) to make a stock solution of 1 mg/ml which was stored at -20 °C until required.

6.3.5 LPS treatment of submerged mono-cultures and of mono- and co-cultures at ALI

LPS from *P. aeruginosa* 50DR (*P. aeruginosa*), *B. cepacia* and *P. aeruginosa* serotype 10 (*P. aeruginosa* 10; obtained from Sigma Aldrich Ltd) were used at 0.1, 1, 10, 100 and 1000ng/ml to treat mono- and co-cultures under submerged conditions or at ALI.

For submerged conditions, cells were seeded onto 24-well plates at a cell density of 1 x 10^5 cells/well in full growth medium. After 24 h incubation under standard conditions the full medium was replaced with serum-free (SF) medium containing ITS supplement (1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin and 0.5 µg/ml sodium selenite; ITS, Sigma), normocin (50mg/ml) and 2mM L-glutamine. For AEM and HPFM, the manufacturer's supplement is added in addition to ITS, as suggested by manufacturer. After another 24 h the ITS-medium is replaced with ITS-medium containing different concentrations of LPS. Four wells were set up for each concentration of LPS and samples were collected 24 h after LPS exposure

for analysis. Cell viability was assessed using CTB as described in 2.4 and Interleukin-8 (IL-8) concentration was measured by ELISA (Peprotech EC Ltd).

The same experimental outline was used for mono- and co-cultures grown at ALI but, based on the results of LPS stimulation of submerged cultures, only the highest concentration of LPS (1000ng/ml) was applied. On these ALI cultures three different challenges were carried for each LPS type. Cells were challenged either from the apical side, from the basal side or from both sides at the same time.

6.3.6 Detection of Interleukin 8 (IL-8) by ELISA

For the quantification of human IL-8 in cell culture supernatants of submerged and ALI mono- and co-cultures treated with live bacteria, a human IL-8 ELISA development kit, was purchased from Peprotech EC Ltd. (London, UK). All reagents were part of the kit unless otherwise stated and were prepared according to manufacturer's product information. IL-8 ELISA protocol is described in detail in 2.19.

6.3.7 Indirect Immunofluorescence staining (flow cytometry)

2 x10^5 cells were washed and incubated with 100µl of primary antibody for 30mins on ice. Cell surface CD14 expression was detected with undiluted 63D3 or the IgG1 isotype control MOPC 21. Following incubation cells were washed twice (PBS/0.1 BSA (w/v) before incubation with 100µl secondary-conjugated antibody (goat anti-mouse PE (1:50) or goat anti-rabbit FITC (1:50)) for at least 30mins on ice. Cells were analysed straight afterwards on the flow cytometer.

6.3.8 Direct Immunofluorescence staining (flow cytometry)

Cell surface TLR4 was determined using direct immunofluorescence staining with PE-conjugated anti-TLR4 antibodies (HTA 125) or IgG2a/K-isotype control. 2 x 10^5 cells, Which were grown submerged in cell culture flasks. Cells were then washed and incubated with 2 µL of HTA 125 or the isotype control for 30mins on ice. Following incubation cells were washed twice with PBS containing 0.1 % BSA (w/v) and analysed straight away using flow cytometry.

6.3.9 Statistical analysis

Throughout this chapter statistical analysis of results and significant differences were determined by 1Way-Anova followed by Tukey's multiple comparison test. Results are presented as mean ± SD. Results were considered significant when $p \leq 0.05$ = *; $p \leq 0.01$ = **; $p \leq 0.001$ = ***.

6.4 Results

6.4.1 Cell viability and IL-8 release of submerged mono-cultures after exposure to LPS

In this chapter all submerged mono-cultures were challenged with three different types of LPS. *B. cepacia* LPS, *P. aeruginosa* 10 LPS and *P. aeruginosa* 50DR LPS were all used in serial dilutions of 0.1, 1, 10, 100, 1000ng/ml. The other mono-and co-cultures were also exposed to these three LPS types but only at 1000 ng/ml.

6.4.1.1 Exposure of submerged HPF mono-cultures to LPS

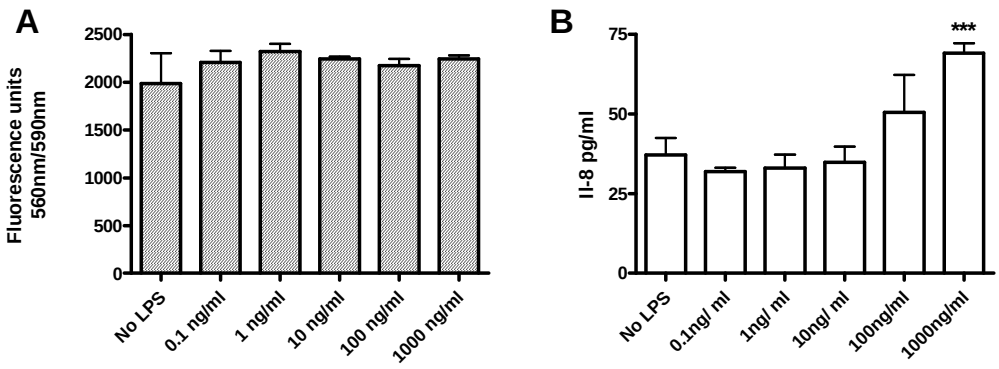

Figure 6.1 Cell viability (A) and IL-8 secretion (B) of HPF after exposure to *B. cepacia* LPS
HPF were seeded onto a 24-well plate at 1 x 10^5 cells per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to serial dilutions of *B. cepacia* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations and cell viability was determined. No effect of any LPS concentration on cell viability was observed. There was no significant stimulation of IL-8 secretion by *B. cepacia* LPS for LPS concentrations 0.1-100 ng/ml. Only 1000 ng/ml *B. cepacia* LPS stimulated a significant increase of IL-8 into the supernatant. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

HPF mono-cultures were grown in 24-well plates and were challenged with serial dilutions of *B. cepacia* LPS for 24 h (figure 6.1). After the incubation with 0.1, 1, 10, 100, 1000ng/ml *B. cepacia* LPS, HPF's cell viability (A) was analysed and found to be constant compared to the control, which had a baseline fluorescence of 1987.13 ± 314.34 FU. Collected supernatants were assayed by ELISA for the presence of IL-8 (B). Of the concentrations of *B. cepacia* LPS tested, only 1000ng/ml induced IL-8 secretion, which increased significantly from 37.17 ± 5.28 pg/ml in the control to 69.06 ± 3.06 pg/ml after LPS challenge.

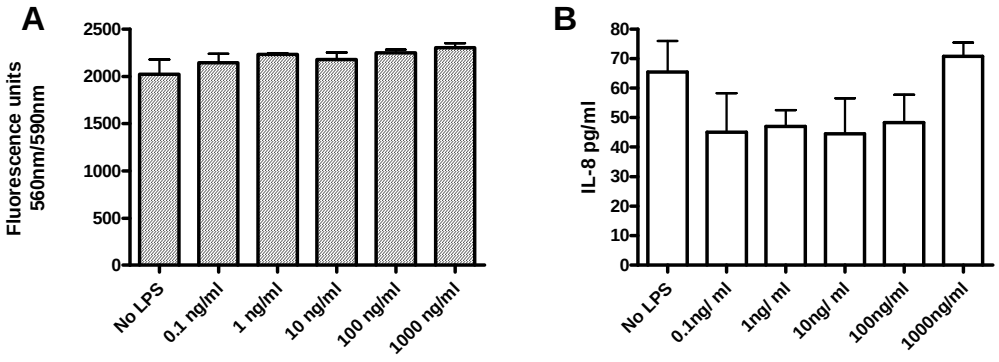

Figure 6.2 Cell viability (A) and IL-8 release (B) of HPF induced by *P. aeruginosa* 10 LPS
HPF were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations by ELISA. HPF's cell viability was determined and no effect of *P. aeruginosa* LPS for any of the concentrations of LPS was found. *P. aeruginosa* LPS did induce IL-8 secretion of this cell line at a concentration of 1000ng/ml. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells per concentration).

HPF were challenged with *P. aeruginosa* 10 LPS at the indicated concentrations. Cell viability (figure 6.2 A) was not changed after 24 h incubation with LPS by any of the concentrations. The control wells had a baseline fluorescence of 1977.26 ± 114.04 FU.IL-8 secretion (figure 6.2 B) was only induced by the highest concentrations of 1000ng/ml of *P. aeruginosa* LPS. In this supernatant the IL-8 concentration was 62.09 ± 5.33 pg/ml compared to 45.98 ± 3.15 pg/ml in the control supernatants.

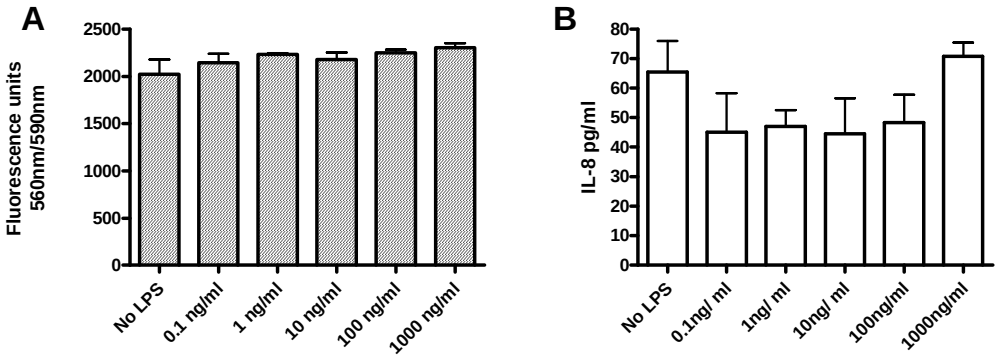

Figure 6.3 Cell viability (A) and IL-8 secretion (B) of HPF after exposure to *P. aeruginosa* 50DR LPS
HPF were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations. HPF's cell viability was determined and no effect of any LPS concentration on cell viability or IL-8 secretion was observed. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

When HPF mono-cultures were challenged with *P. aeruginosa* 50DR LPS (figure 6.3) under submerged conditions, neither the cell viability (A) nor the IL-8 secretion (B) was changed in response to the induction with LPS. The baseline fluorescence measured for the control mono-cultures was 2018.84 ± 156.48 FU after the challenge. The observed IL-8 concentration was was 65.45 ± 10.58 pg/ml for the control wells.

6.4.1.2 Exposure of submerged C38 mono-culture to LPS

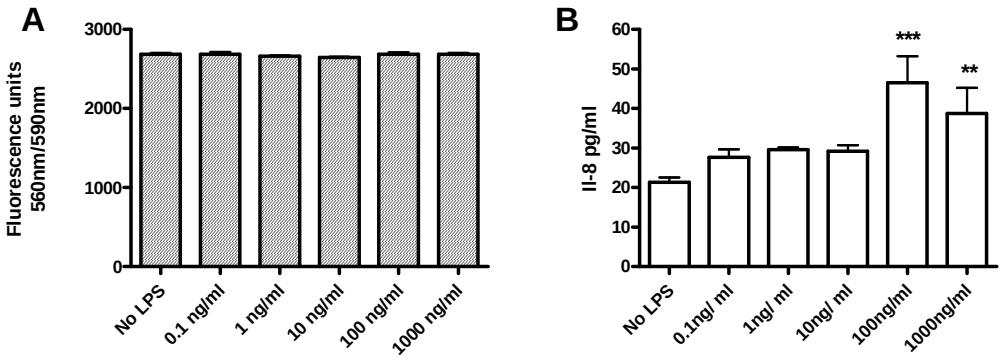

Figure 6.4 Cell viability (A) and IL-8 secretion (B) of C38 challenged with *B. cepacia* LPS
C38 were grown to confluence on a 24-well plate and incubated for 24 h before they were serum starved for 24 h. Afterwards C38 were exposed to serial dilutions of *B. cepacia* LPS for 24 h. Supernatants were collected and assayed by ELISA for the presence of IL-8. Cell viability was determined and no effect could be observed for any of the LPS concentrations, when compared to the control, which was C38 with medium only. 100ng/ml as well 1000ng/ml of *B. cepacia* LPS induced significant IL-8 secretion. Each bar represents the mean ± SD from 3 seperate experiments (each experiment quadruplicate wells).

After C38 submerged mono-cultures were challenged with different concentrations of *B. cepacia* LPS (0.1, 1, 10, 100, 1000ng/ml), cell viability (A) was analysed and no significant changes were observed for any of the LPS concentrations (baseline fluorescence = 2687.05 ± 14.76 FU) (figure 6.4). IL-8 secretion (B), however was significantly increased after 24 h incubation with 100 ng/ml, the IL-8 concentration increased to 46.5 ± 6.75 pg/ml and with 1000ng/ml it increased to 38.77 ± 6.43 pg/ml compared to the control, which had an IL-8 concentration of 21.39 ± 1.25 pg/ml.

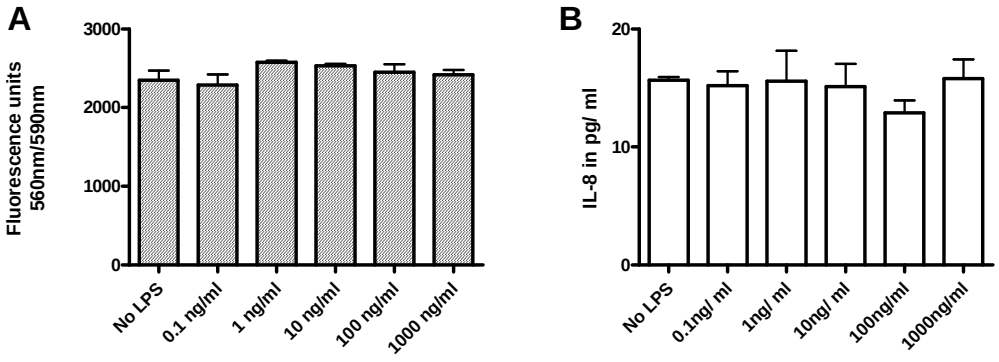

Figure 6.5 Cell viability (A) and IL-8 release (B) of C38 induced with *P. aeruginosa* 10 LPS
C38 were grown to confluence on a 24-well plate and incubated for 24 h before they were serum starved for 24 h. Afterwards C38 were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and assayed by ELISA for the presence of IL-8. Cell viability was determined and no effect could be observed for any of the LPS concentrations, when compared to the control, which was C38 with medium only. 100ng/ml as well 1000ng/ml of *P. aeruginosa* LPS induced no significant increases in IL-8 secretion. Each bar represents the mean ± SD from 3 seperate experiments (each experiment quadruplicate wells).

When submerged C38 mono-cultures were exposed to different concentrations of *P. aeruginosa* 10 LPS, no effects on the cell viability (figure 6.5 A) were observed after 24 h incubation. The control mono-cultures of C38, which were exposed to SF-growth medium only, showed a baseline fluorescence of 2352.48 ± 118.25 FU. *P. aeruginosa* 10 LPS did not increase IL-8 secretion (figure 6.5 B) at any of the concentrations employed. For the control the IL-8 concentration observed was 15.64 ± 0.29 pg/ml.

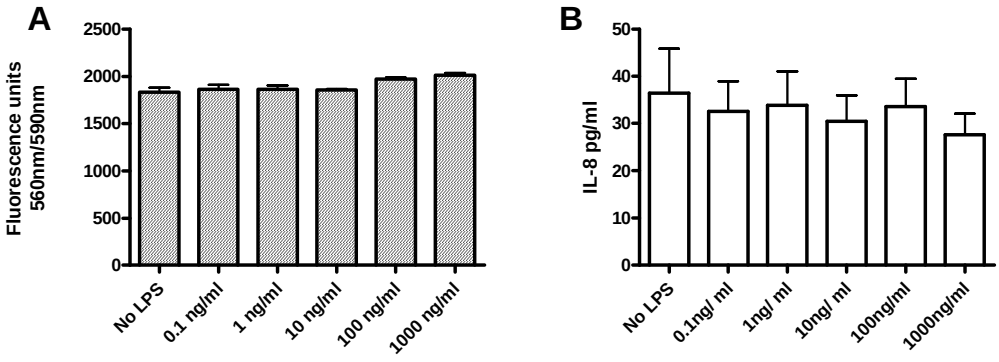

Figure 6.6 Cell viability (A) and IL-8 secretion (B) of C38 after exposure to *P. aeruginosa* 50DR LPS
C38 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards C38 were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. S All supernatants were collected and analysed for IL-8 concentrations and C38's cell viability was determined. For both assays no effect of different LPS concentration were identified Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells)

Challenging submerged C38 mono-cultures with LPS from *P. aeruginosa* 50DR gave similar data to *P aeruginosa* 10. No changes in cell viability (figure 6.6 A) in response to any of the LPS concentrations were seen, compared to baseline fluorescence of 1833.28 ± 44.51 FU, measured for the control. After 24 h incubation *P. aeruginosa* 50DR LPS did not induce an increase in IL-8 secretion (figure 6.6 B), which was measured in the cell culture supernatants. Control mono-cultures secreted 36.45 ± 9.36 pg/ml.

6.4.1.3 Exposure of submerged IB3-1 mono-cultures to LPS

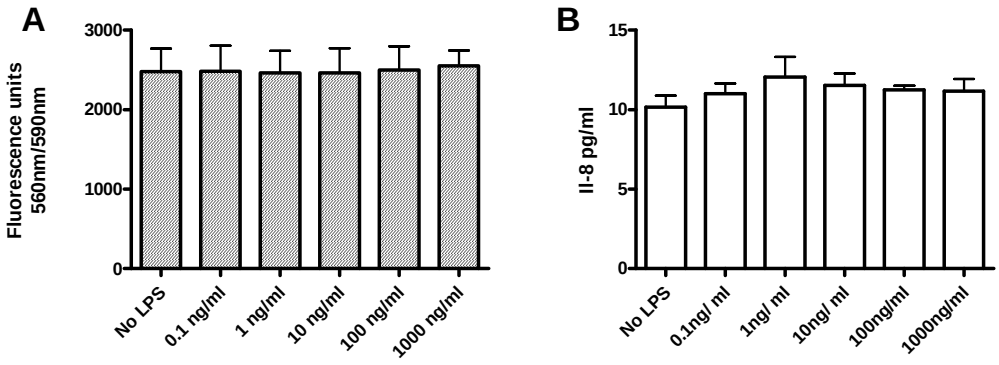

Figure 6.7 Cell viability (A) and IL-8 secretion (B) of IB3-1 after exposure to *B. cepacia* LPS
IB3-1 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to serial dilutions of *B. cepacia* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations by ELISA. IB3-1's cell viability was determined and no effect of *B. cepacia* LPS for any of the concentrations of LPS was found. *B. cepacia* LPS did not stimulate IL-8 secretion by this cell line. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

When IB3-1 were challenged with different dilutions of *B. cepacia* LPS the cell viability (figure 6.7 A) was not significantly affected compared to the control, which had a baseline fluorescence of 2479.79 ± 290.01 FU. IL-8 concentrations (figure 6.7 B) in the collected supernatants were assayed and no IL-8 secretion was observed to be induced, when compared to the control, which had an IL-8 concentration of 10.15 ± 0.75 pg/ml.

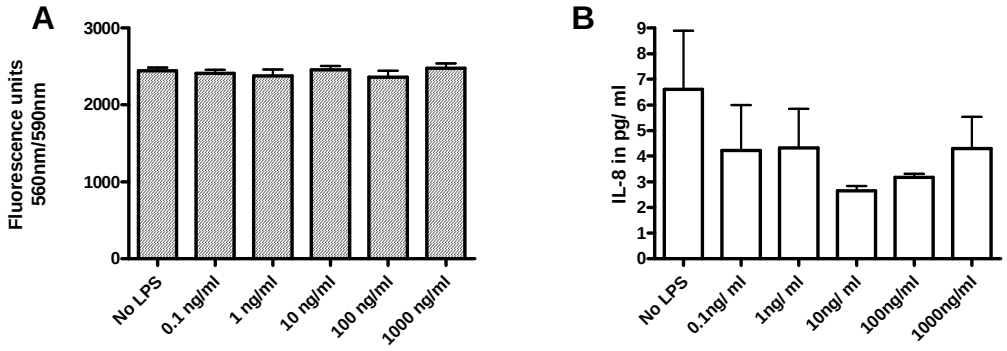

Figure 6.8 Cell viability (A) and IL-8 release (B) of IB3-1 after exposure to *P. aeruginosa* 10 LPS
IB3-1 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations by ELISA. IB3-1's cell viability was determined and no effect of *P. aeruginosa* LPS for any of the concentrations of LPS was found. *P. aeruginosa* LPS did not stimulate IL-8 secretion in this cell line. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

IB3-1 mono-cultures were grown under submerged conditions and were then exposed to the indicated concentrations of *P. aeruginosa* 10 LPS for 24 h. IB3-1's cell viability (figure 6.8 A) was not influenced at all, when compared to the control (baseline FU 2440.06 ± 44.96). Neither were the IL-8 concentrations affected by this event, which was 6.60 ±2.29 pg/ml in the supernatants of the control (figure 6.8 B).

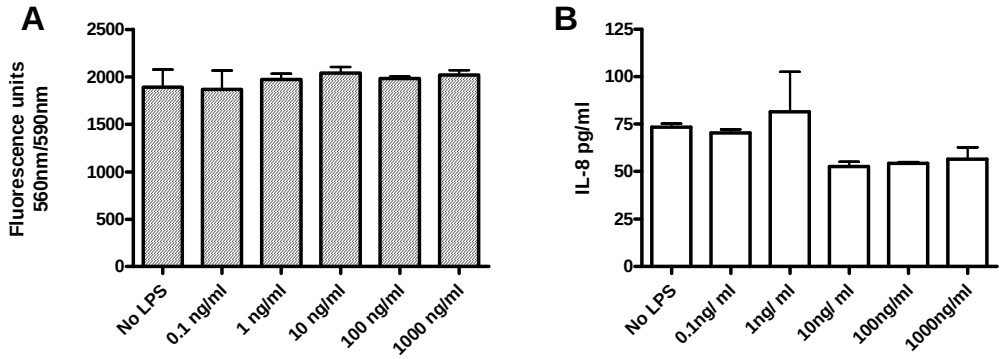

Figure 6.9 Cell viability (A) and IL-8 secretion (B) of IB3-1 after induction by *P. aeruginosa* 50DR LPS
IB3-1 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards the cells were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and analysed for IL-8 concentrations. IB3-1's cell viability was examined and no effect of different LPS concentration on cell viability or IL-8 secretion was observed. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

IB3-1 mono-cultures were grown under submerged conditions and were this time challenged with *P. aeruginosa* 50 DR LPS at the indicated LPS concentrations but no significant changes were observed, when examining cell viability (figure 6.9 A) and IL-8 release (figure 6.9 B) in the collected supernatants. The baseline fluorescence that was measured for control wells was 1889.93 ± 188.07 FU and the IL-8 concentration found in control wells was 73.35 ± 2.00 pg/ml

6.4.1.4 Exposure of submerged Calu-3 mono-cultures

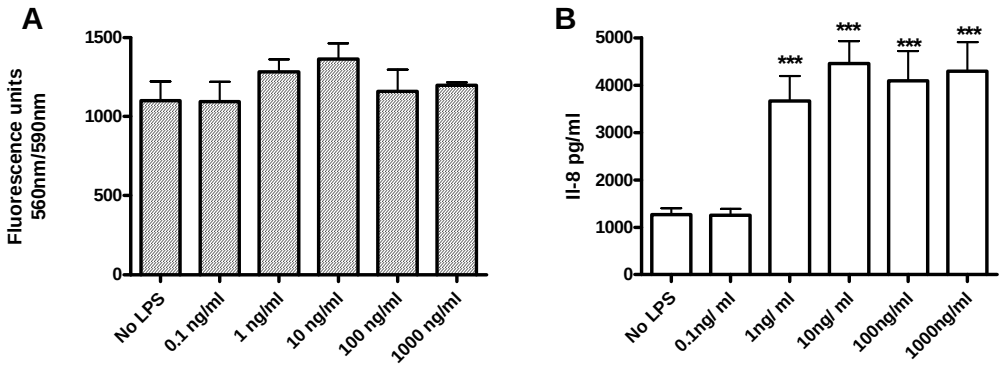

Figure 6.10 Cell viability (A) and IL-8 secretion (B) of Calu-3 induced with *B. cepacia* LPS
Calu-3 were grown to confluence on a 24-well plate and incubated for 24 h before they were serum starved for 24 h. Afterwards Calu-3 were exposed to serial dilutions of *B. cepacia* LPS for 24 h. Supernatants were collected and assayed by ELISA for the presence of IL-8. Cell viability was determined and no effect could be observed for any of the LPS concentrations, when compared to the control, which was Calu-3 with medium only. Apart from 0.1 ng/ml *B. cepacia* LPS, all other concentrations of LPS tested induced a significant increase in IL-8 secretion compared to control. Each bar represents the mean ± SD from 3 seperate experiments (each experiment quadruplicate wells).

B. cepacia LPS was used in the indicated concentrations to challenge submerged Calu-3 mono-cultures. Cell viability (figure 6.10 A) of this mono-culture was not influenced by exposure to *B. cepacia* LPS for 24 h incubation, when compared to control wells, which showed a baseline fluorescence of 1100.39 ± 122.36 FU. IL-8 secretion (figure 6.10 B), however was induced by LPS concentrations higher than 0.1 ng/ml. Control supernatants had an IL-8 concentration of 1265.48 ± 138.66 pg/ml, which increased to 3664.67 ± 525.27 pg/ml with an LPS concentration of 1 ng/ml and to 4455.77 ± 470.04 pg/ml with LPS used at 10 ng/ml. When LPS was used at 100 ng/ml or 1000ng/ml the IL-8 concentration increased to a similar level with 4094.44 ± 622.27 pg/ml and 4294.31 ± 610.60 pg/ml, respectively.

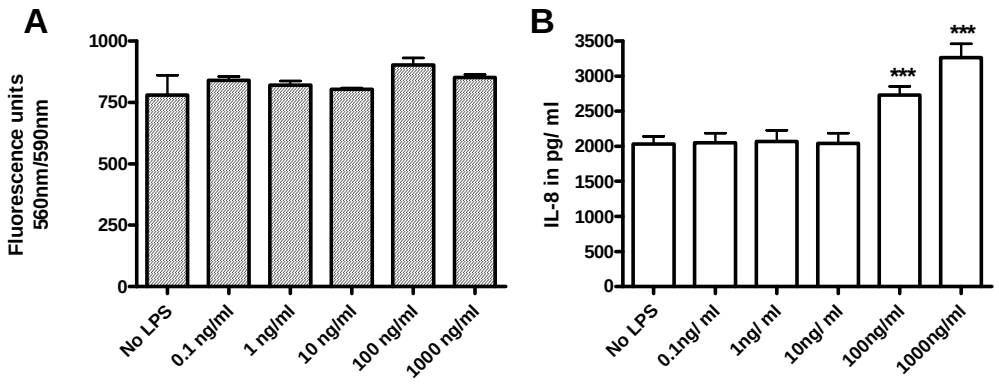

Figure 6.11 Cell viability (A) and IL-8 secretion (B) of Calu-3 induced with *P. aeruginosa* 10 LPS
Calu-3 were grown to confluence on a 24-well plate and incubated for 24 h before they were serum starved for 24 h. Afterwards Calu-3 were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and assayed by ELISA for the presence of IL-8. Cell viability was determined and no effect could be observed for any of the LPS concentrations, when compared to the control, which was Calu-3 with medium only. Only 100ng/ml and 1000ng/ml induced significant increase in IL-8 secretion by Calu-3 mono-culture. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

Submerged Calu-3 mono-cultures did not show any changes in cell viability (figure 6.11 A) when this was analysed after 24 h incubation with several different concentrations of *P. aeruginosa* 10 LPS. Control mono-cultures, which were exposed only to SF-growth medium produced a baseline fluorescence of 780.10 ± 81.61 FU. IL-8 secretion (figure 6.11 B) by Calu-3 mono-cultures was induced at a concentration of 100 ng/ml (2734.89 ± 115.95 pg/ml) as well as at 1000 ng/ml (3264.48 ± 196.09 pg/ml) compared to control (2032.74 ± 109.1 pg/ml).

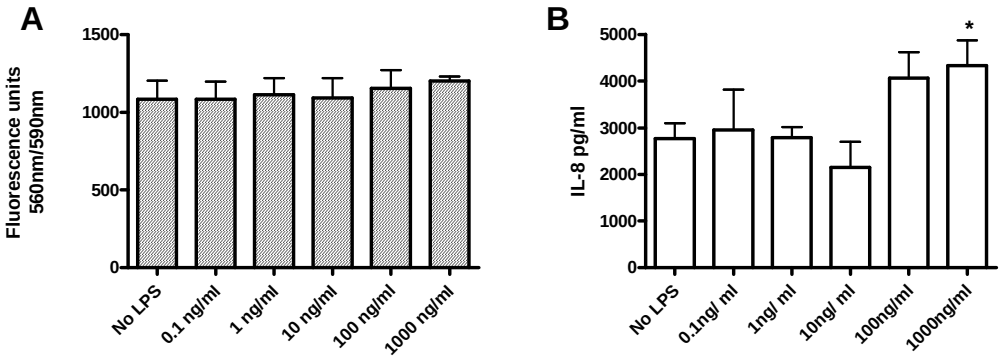

Figure 6.12 Cell viability (A) and IL-8 secretion (B) of Calu-3 induced with *P. aeruginosa* 50DRLPS
Calu-3 were grown to confluence before they were serum starved for 24 h. Afterwards Calu-3 were exposed to serial dilutions of *P. aeruginosa* LPS for 24 h. Supernatants were collected and assayed by ELISA for the presence of IL-8. Cell viability was determined and found to be unchanged, when compared to the control, which was Calu-3 with medium only. IL-8 secretion was induced by 1000ng/ml LPS of *P. aeruginosa*. Each bar represents the mean ± SD from 3 separate experiments (each experiment quadruplicate wells).

For Calu-3 mono-cultures, the cell viability (figure 6.12 A) was found to be constant after the exposure to *P. aeruginosa* 50DR LPS, compared to the control, which showed 1083.14 ± 119.44 FU when measured after the challenge. IL-8 secretion (figure 6.12 B), however, was significantly increased by an LPS concentration of 1000 ng/ml and reached 4325.58 ± 552.54 pg/ml. The control supernatant contained 2767.52 ± 325.92 pg/ml of IL-8.

6.4.2 Cell viability and IL-8 release of mono-cultures at ALI after exposure to LPS

6.4.2.1 Exposure of submerged HPF mono-cultures on TWs to LPS

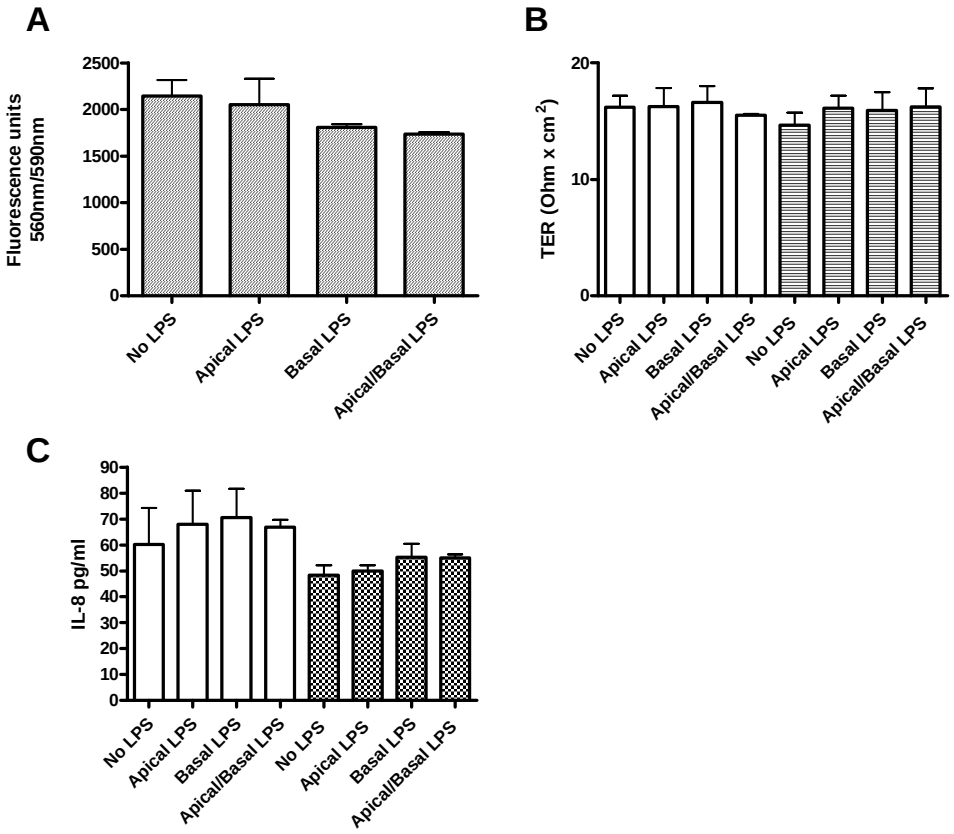

Figure 6.13 Cell viability (A), TER (B) and IL-8 secretion (C) of HPF after exposure to *B. cepacia* LPS
HPF were grown under submerged conditions on TWs for 14 days before these were challenged with 1000ng/ml *B. cepacia* LPS, which was applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs, which were only exposed to HPFM medium. Cell viability of HPF mono-culture was not affected by the presence of *B. cepacia* LPS and nor was the TER, which was measured before (clear bars) and directly after (striped bar) the 24 h incubation with *B. cepacia* LPS. Supernatants were analysed for IL-8 concentrations but no significant differences were observed in apical supernatants (clear bars) or basolateral medium (checked bars), compared to the control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF mono-cultures were grown under submerged conditions on TWs for 14 days before they were challenged with *B. cepacia* LPS. The control TWs were treated the same way as all others but were exposed to SF-growth medium only, whereas the TWs that were challenged with LPS were exposed to this from either the apical side, the basal side or they were exposed to LPS on both sides at the same time. HPF's cell viability (figure 6.13 A) was not changed after 24 h incubation with 1000ng/ml *B. cepacia* LPS, when compared to the control, for which a baseline fluorescence of 2478.22 ± 758.96 FU was measured. This was also found for TER (figure 6.13 B) measured before (clear bars) and after (striped bars) the challenge. The control cultures had a TER of 16.20 ± 0.33 Ω x cm^2 before the exposure to LPS and a TER of 16.43 ± 0.34 Ω x cm^2 after the challenge. IL-8 release from HPF mono-cultures was not increased significantly compared to control (figure 6.13 C). This was true for IL-8 was measured after the challenge in apical supernatants (clear bars) as well as in the basolateral medium (checked bars). The control cultures of HPF had 225.87 ± 58.75 pg/ml IL-8 in the apical supernatant and 230.41 ± 34.2 pg/ml IL-8 in the basolateral medium.

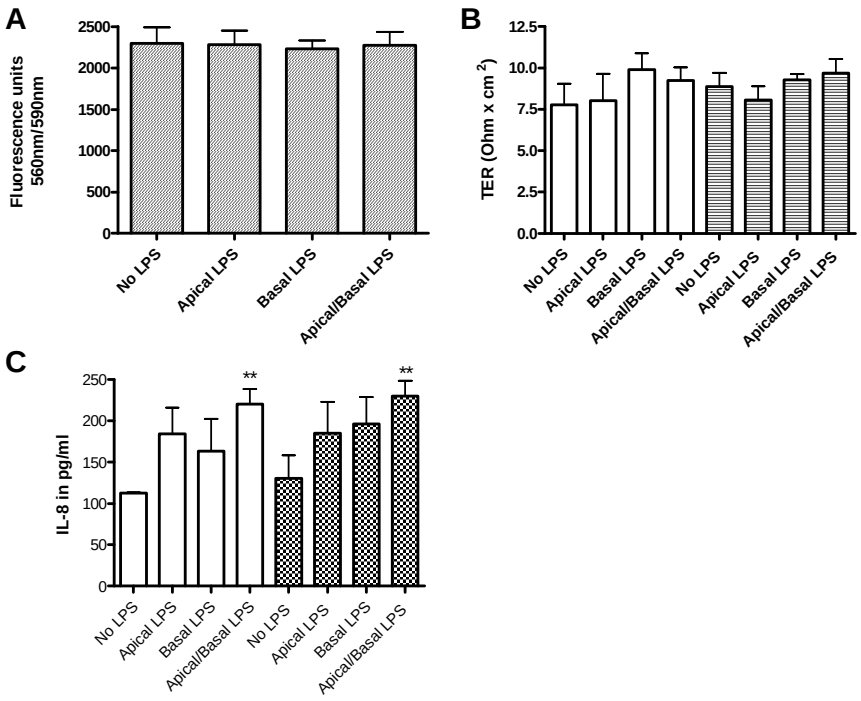

Figure 6.14 Cell viability (A) TER (B) and IL-8 release (C) of HPF mono-culture after exposure to *P. aeruginosa 10* LPS
HPF mono-cultures were grown submerged on TWs for 14 days before these cells were exposed to *P. aeruginosa 10* LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability after 24 h exposure to LPS was not significantly affected compared to the control TWs, which were exposed to growth medium only. TER was measured before (clear bars) and after (striped bars) the experiment but no significant difference was observed after 24 h incubation with *P. aeruginosa 10* LPS. IL-8 release of HPF mono-culture, however, was significantly increased when LPS was applied simultaneously on both sides. This was seen in the apical supernatants (clear bars) as well as in the basal medium samples (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Mono-cultures of HPF were exposed to *P. aeruginosa* 10 LPS after they have been cultured under submerged conditions for 14 days on TWs. After the TWs were challenged with 1000 ng/ml *P. aeruginosa* 10 LPS from either the apical side, the basolateral side or from both sides simultaneously the cell viability (figure 6.14 A) was assayed and was found to be unaffected by these events, when compared to the control's baseline fluorescence, which was 2297.08 ± 193.45 FU. HPF mono-cultures were also monitored in terms of changes in electrical resistance and the TER (figure 6.14 B) was measured before (clear bars) and after (striped bars). No significant changes were observed for any of the challenges. Here the control TWs had a TER of 7.77 ± 1.29 Ω x cm^2 before the exposure to *P. aeruginosa* 10 LPS and 8.87 ± 0.83 Ω x cm^2 after the challenge. Furthermore the IL-8 concentrations (figure 6.14 C) in apical supernatants (clear bars) and basal medium samples (checked bars) was analysed. The exposure to *P. aeruginosa* 10 LPS induced a significant increase of IL-8 in apical supernatants of TWs that were challenged

from both sides at the same time. This increase was seen in the apical supernatant (220.14 ± 18.25 pg/ml) as well as in the basolateral medium (229.61 ± 18.98 pg/ml). The control TWs had 112.65 ± 0.79 pg/ml of IL-8 in the apical supernatants and 130.30 ± 28.22 pg/ml of IL-8 in the basolateral medium.

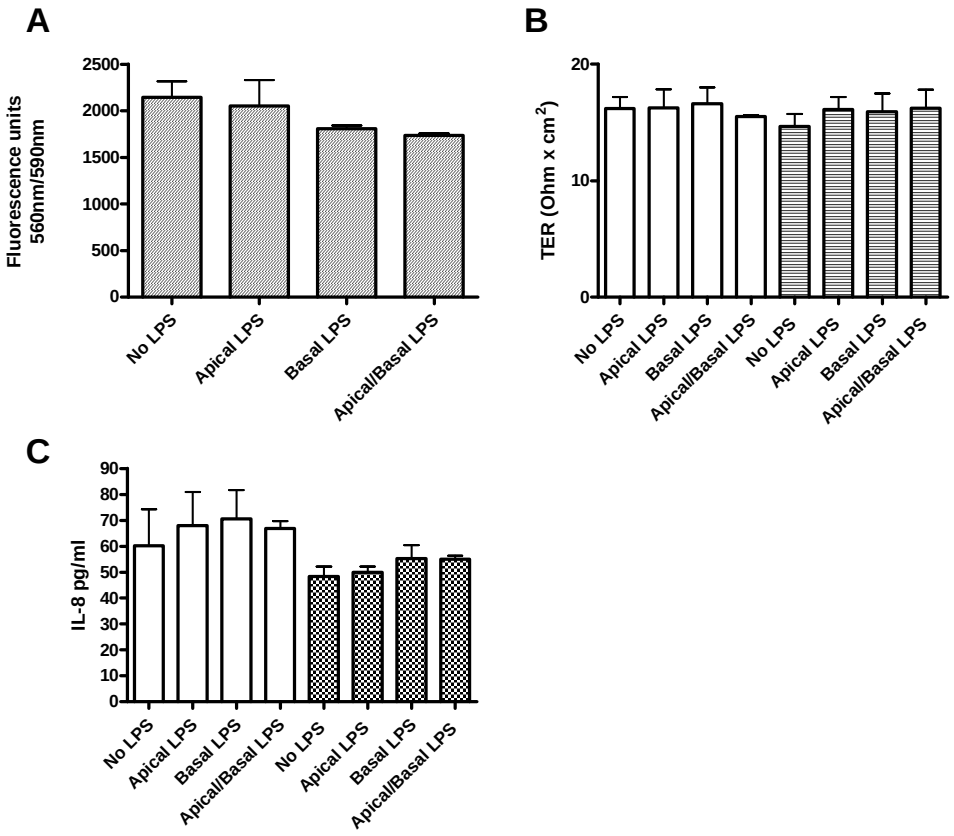

Figure 6.15 Cell viability (A) TER (B) and IL-8 release (C) of HPF mono-culture after exposure to *P. aeruginosa* 50DR LPS
HPF mono-cultures were grown submerged on TWs for 14 days before these cells were exposed to *P. aeruginosa* LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability after 24 h exposure to LPS was not significantly affected compared to the control TWs, which were exposed to growth medium only. TER and IL-8 concentratione were also examined and found to be unchanged. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF mono-cultures were grown on TWs for 14 days under submerged conditions before these were used to be exposed to the third type of LPS under investigation, which was from the clinical isolate of *P. aeruginosa* 50DR LPS was used at 1000 ng/ml and was applied either apically, basolaterally or on both sides at the same time. Cell viability (figure 6.15 A), TER (figure 6.15 B) and IL-8 concentrations (figure 6.15 C) in apical (clear bars) and basolateral samples (checked bars) were analysed. None of the measured parameters were influenced by *P. aeruginosa* 50DR LPS after 24 h incubation. For control cultures of HPF at ALI a baseline fluorescence of 2146.73 ±

171.28 FU was measured. The TER for the control was 16.20 ± 0.99 Ω x cm^2 before (clear bars) the challenge and 14.67 ± 1.08 Ω x cm^2 afterwards (striped bars). The IL-8 concentrations of these controls were 60.17 ± 14.22 pg/ml in the apical supernatant and 48.30 ± 3.88 pg/ml in the basolateral medium.

6.4.2.2 Exposure of C38 mono-culture at ALI to LPS

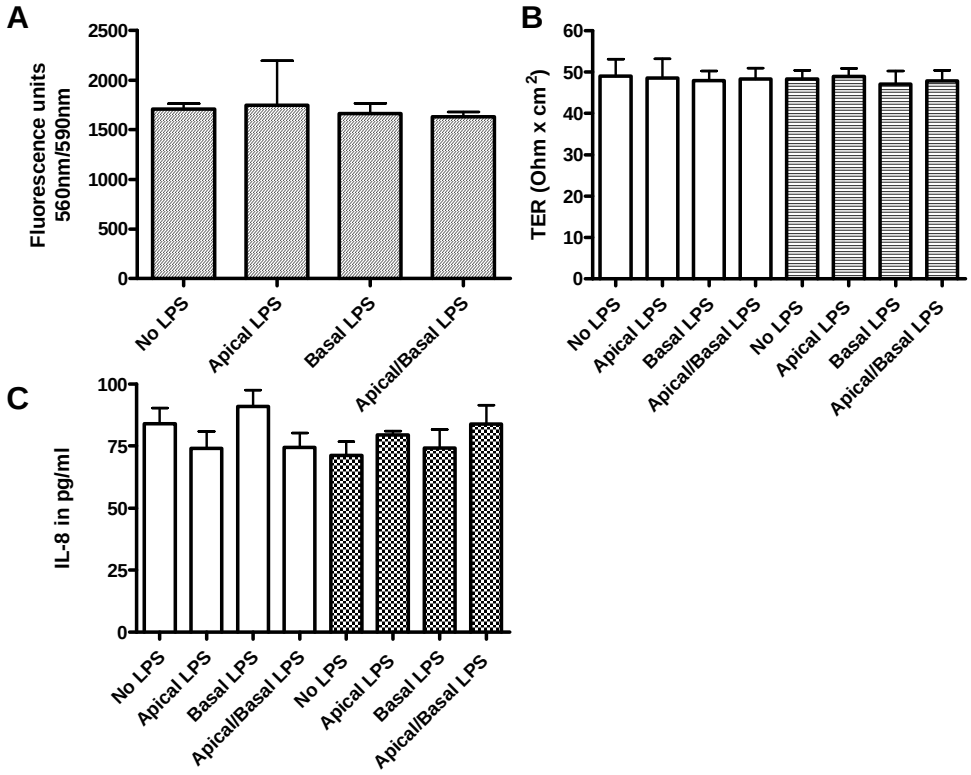

Figure 6.16 Cell viability (A), TER (B) and IL-8 secretion (C) of C38 at ALI after exposure to *B. cepacia* LPS
C38 were grown on TWs for 14 days before these were challenged with 1000ng/ml *B. cepacia* LPS, which was applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs, which were only exposed to AEM medium. Cell viability of C38 mono-culture was not affected by the presence of *B. cepacia* LPS and nor was the TER, which was measured before (clear bars) and directly after (striped graphs) the 24 h incubation with *B. cepacia* LPS. Supernatants were analysed for IL-8 concentrations but no significant differences were observed in apical supernatants (clear bars) or basolateral medium (checked bars), compared to the control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38 mono-cultures that were challenged with 1000ng/ml of *B. cepacia* LPS did not show any change in cell viability (figure 6.16 A) after exposure to LPS for 24 h, which was either applied apically, basolaterally or on both sides simultaneously. Baseline fluorescence of control cultures was 1707.47 ± 96.68 FU. Exposure of C38 mono-cultures to *B. cepacia* LPS did not have a direct

influence on the TER (figure 6.16 B) when comparing the TER before (clear bars) and the TER after the challenges (striped bars). TER measured for the control was 49.09 ± 4.06 Ω x cm^2 before the challenge and it was 48.44 ± 1.99 cm^2 after the challenge. The third aspect that was analysed was IL-8 concentration (figure 6.16 C) and was measured in all apical supernatants (clear bars) as well as in all basolateral medium samples (checked bars). IL-8 concentrations were not affected by any of the three different approaches of exposure, when compared to the control, which had an IL-8 concentration of 84.02 ±11.08 pg/ml in the apical supernatant and 71.24 ± 9.75 pg/ml in the basolateral medium.

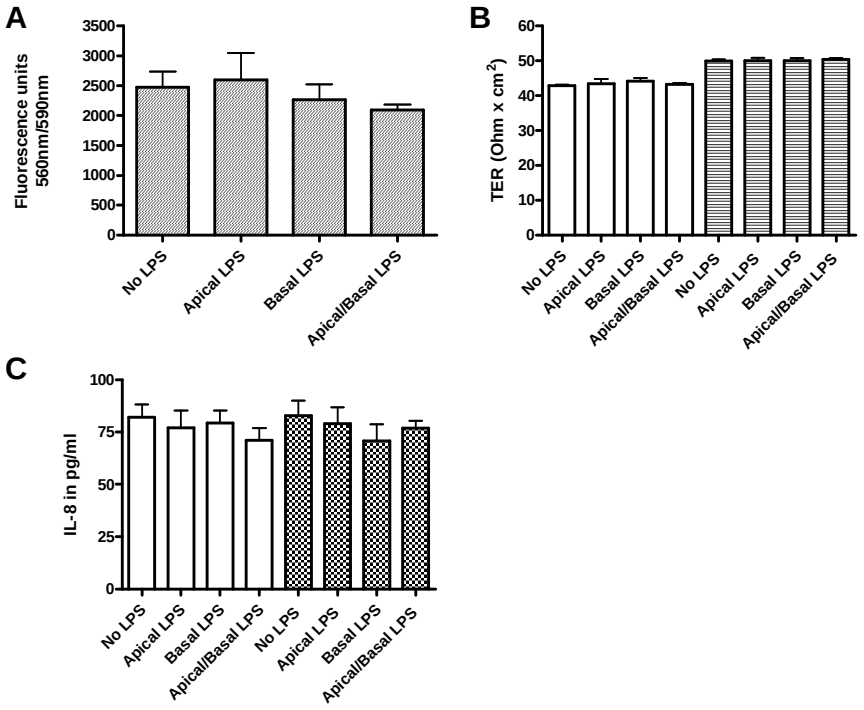

Figure 6.17 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to LPS of *P. aeruginosa* 10
C38 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa 10* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A), TER (B) or IL-8 concentrations (C) were all found to be unchanged compared to applicable controls throughout this experiment. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38 mono-cultures at ALI were challenged with 1000 ng/ml *P. aeruginosa* 10 LPS after 14 days of culture. LPS was applied either apically, basolaterally or simultaneously from both sides and cells were exposed for 24 h. *P. aeruginosa* 10 LPS did not influence any of the measured parameters. Cell viability (figure 6.17 A) of all mono-cultures analysed was comparable to the viability found

in control TWs (2472.67 ± 265.50 FU). TER values (figure 6.17 B) measured were not changed significantly by any of the three challenges when comparing TERs from afterwards (striped bars) to the corresponding one measured beforehand (clear bars). For the control TWs a TER of 42.91 ± 0.22 Ω x cm^2 was measured before and a TER of 49.87 ± 0.44 Ω x cm^2 was measured afterwards. Secretion of IL-8 (figure 6.17 C) among all samples assayed was similar, even when comparing apical supernatants (clear bars) to basolateral medium samples. For control cultures an IL-8 concentration of 82.25 ± 5.95 pg/ml was observed in the apical supernatant and an IL-8 concentration of 82.91 ± 7.10 pg/ml was measured in the basolateral medium.

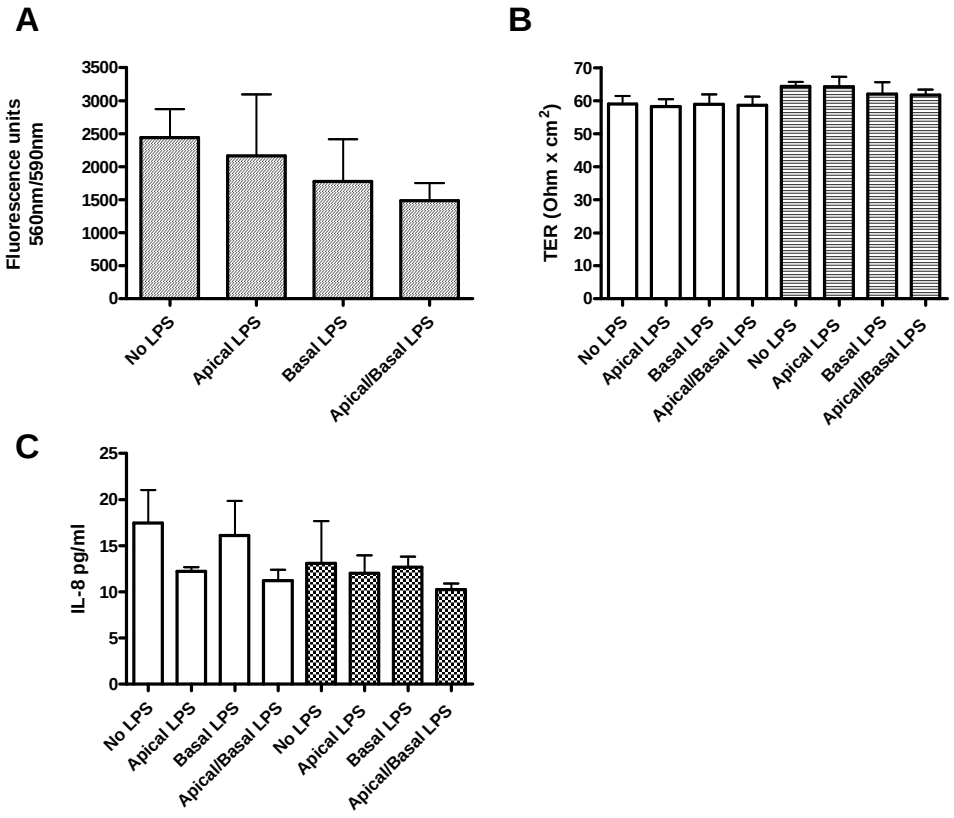

Figure 6.18 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to LPS of *P. aeruginosa* 50DR

C38 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A), TER (B) or IL-8 concentrations were all found to be unaffected compared to applicable controls throughout this experiment. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Cell viability (figure 6.18 A) of C38 mono-cultures at ALI, which were apically, basolaterally or simultaneously challenged, on both sides with *P. aeruginosa* 50DR LPS for 24 h, was not affected. The FU observed for the challenged mono-cultures of C38 were similar to the control, which was 2440.13 ± 432.55 FU. TER (figure 6.18 B) was also monitored and was measured before (clear bars) and after (striped bars) all three different ways of treatment. The TER of the control was 59.03 ± 2.44 Ω x cm^2 before the challenge and it was 64.35 ± 1.35 Ω x cm^2 afterwards and was not significantly different after any of the different exposures to LPS. IL-8 concentrations (figure 6.18 C) were assayed after exposure to *P. aeruginosa* 50DR

LPS in apical supernatants (clear bars) as well as in basolateral medium samples (checked bars) and no significant differences were observed, when compared to the control, which had 17.46 ± 3.57 pg/ml in the apical supernatant and 13.12 ± 4.53 pg/ml in the basolateral medium.

6.4.2.3 Exposure of IB3-1 mono-cultures at ALI to LPS

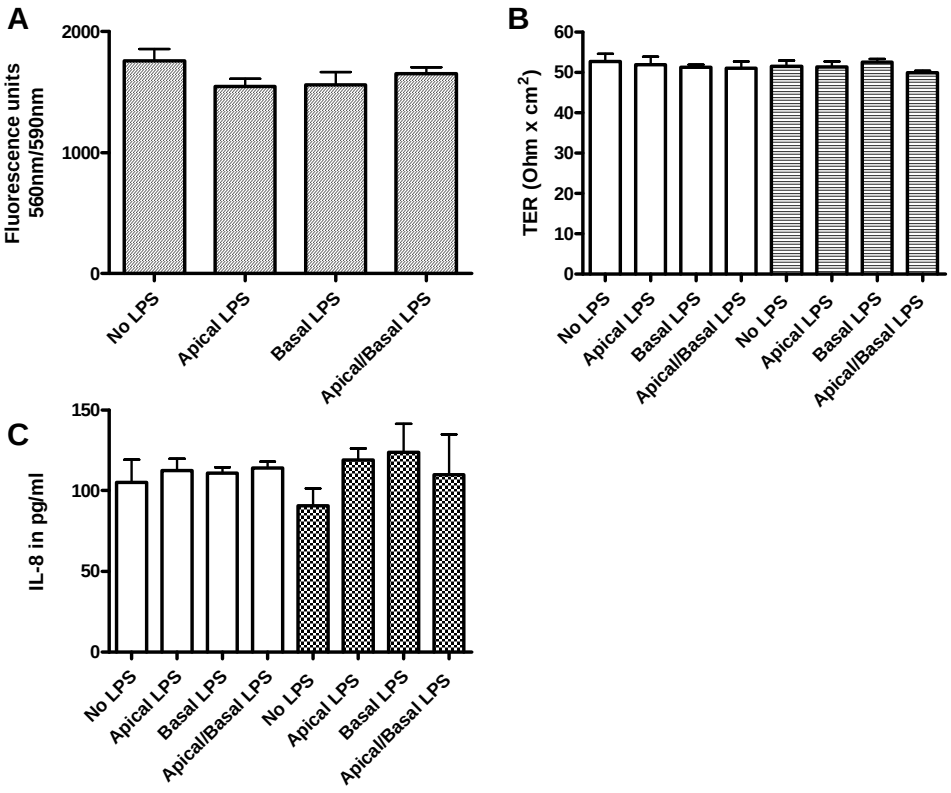

Figure 6.19 Cell viability (A), TER (B) and IL-8 secretion (C) of IB3-1 at ALI after exposure to *B. cepacia* LPS IB3-1 were grown on TWs for 14 days before these were challenged with 1000ng/ml *B. cepacia* LPS, which was applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs, which were only exposed to AEM medium. Cell viability of IB3-1 at ALI was not affected by the presence of *B. cepacia* LPS, nor was TER or IL-8 secretion after 24 h incubation with *B. cepacia* LPS, compared to the appropriate control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The cell viability (figure 6.19 A) of IB3-1 mono-cultures at ALI did not show any significant changes after 24 h exposure to *B. cepacia* LPS, when compared to the baseline fluorescence of the control (1759.40 ± 169.24 FU). TER (figure 6.20 B) was monitored before (clear bars) and after (striped bars) the challenges but did not show any significant differences to the resistance measured in control TWs, which was 52.73 ± 1.9 Ω x cm^2 before the challenge and 51.6 ± 1.34 Ω x cm^2 after afterwards. IL-8 concentrations (figure 6.19 C) of challenged TWs were observed to be similar to the control (apical supernatant: 105.25 ± 24.22 pg/ml, basolateral medium: 90.54 ± 18.85 pg/ml)

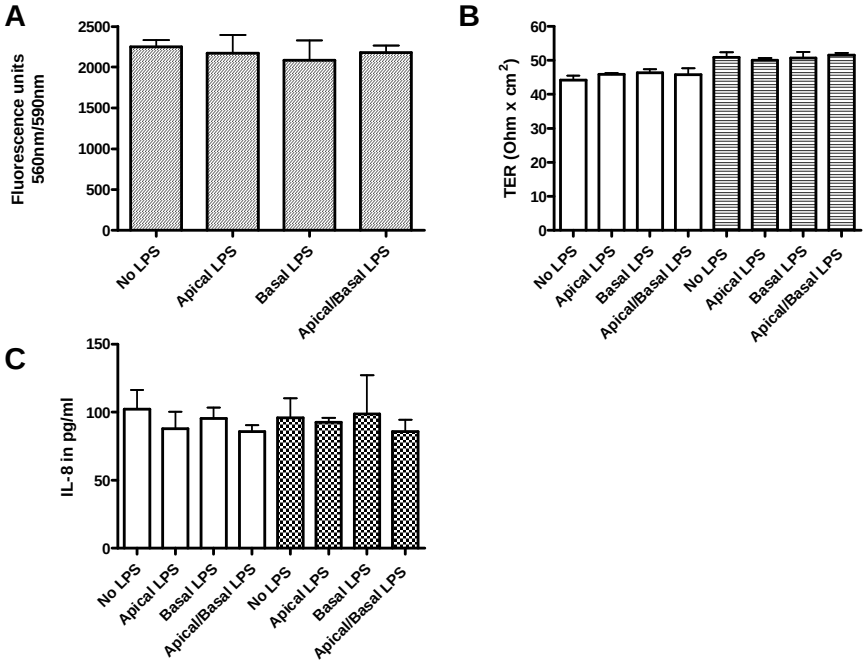

Figure 6.20 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 mono-cultures after exposure to LPS of *P. aeruginosa* 10
IB3-1 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa 10* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A), TER (B) and IL-8 concentrations were examined and all found to be unchanged compared to applicable controls throughout this experiment. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

P. aeruginosa 10 LPS was used at a concentration of 1000 ng/ml to challenge IB3-1 mono-cultures at ALI. Three different approaches were carried out and TWs were challenged either apically, basolaterally or from both compartments at the same time. Cell viability (figure 6.20 A) was not influenced by the exposure to *P. aeruginosa* 10 LPS and was similar for all measured TWs, including the control (2248.89 ± 84.63 FU). TER (figure 6.20 B) was monitored before (clear

bars) and after (striped bars) the challenges and no change in electrical resistance was observed, when comparing these to the control (44.22 ± 1.27 Ω x cm^2 before, 50.86 ± 1.50 Ω x cm^2 afterwards). Apical supernatants and basolateral medium was collected of each TW and analysed for IL-8 concentrations (figure 6.20 C). In the apical supernatant (clear bars) of the control there was 102.15 ± 14.32 pg/ml of IL-8 and 95.86 ± 14.29 pg/ml was observed in the basolateral medium.

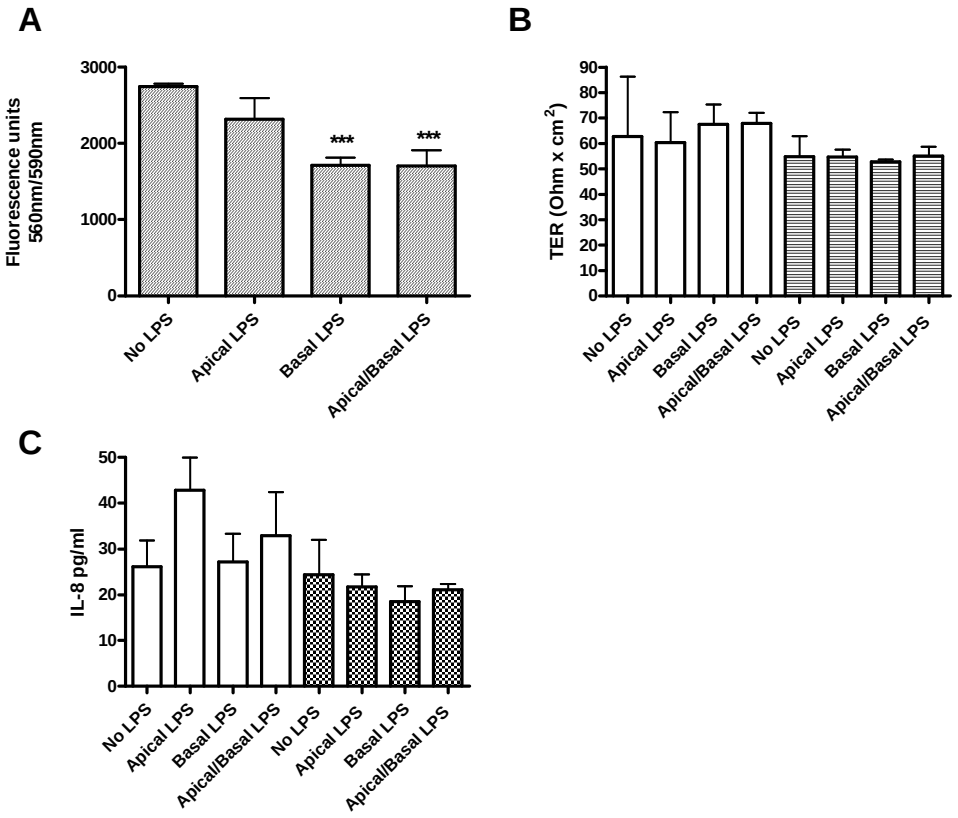

Figure 6.21 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 mono-cultures after exposure to LPS of *P. aeruginosa* 50DR
IB3-1 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A) was analysed and was significantly decreased when LPS was applied either basolaterally or from both sides at the same time. TER (B) and IL-8 concentrations (C) were also examined and these were found to be unaffected compared to applicable controls throughout this experiment. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

LPS from *P. aeruginosa* 50DR was used at 1000 ng/ml to treat mono-cultures of IB3-1 grown at ALI for 14 days. LPS was applied either apically, basolaterally or simultaneously on both sides and cells were exposed for 24 h before samples were collected to be assayed for cell viability (figure

6.21 A). IB3-1's cell viability was significantly reduced when cells were challenged basolaterally or from both sides at the same time. After 24 h exposure to basal medium containing LPS the cell viability showed a signal of only 1711.41 ± 105.41 FU compared to 2742.35 ± 42.20 FU in the control samples. When challenged from both sides simultaneously the result was similar with 1703.94 ± 207.46 FU. However, TER (figure 6.21 B) measured before (clear bars) and after (striped bars) these three challenges was not changed significantly after 24 h incubation with LPS. TER measured for the control was 62.77 ± 23.56 Ω x cm^2 before the exposure to LPS and 54.85 ± 8.05 Ω x cm^2 afterwards. Furthermore IL-8 concentrations (figure 6.21 C) were measured in all apical supernatants (clear bars) and in all basolateral medium samples (checked bars) after the three different approaches of LPS exposure and no significant differences in concentration were found for any of the challenges, when compared to IL-8 concentrations found in the control. In the apical supernatant there was 26.15 ± 5.67 pg/ml of IL-8 and in the basolateral compartment there was 24.36 ± 7.57 pg/ml.

6.4.2.4 Exposure of Calu-3 mono-cultures to LPS

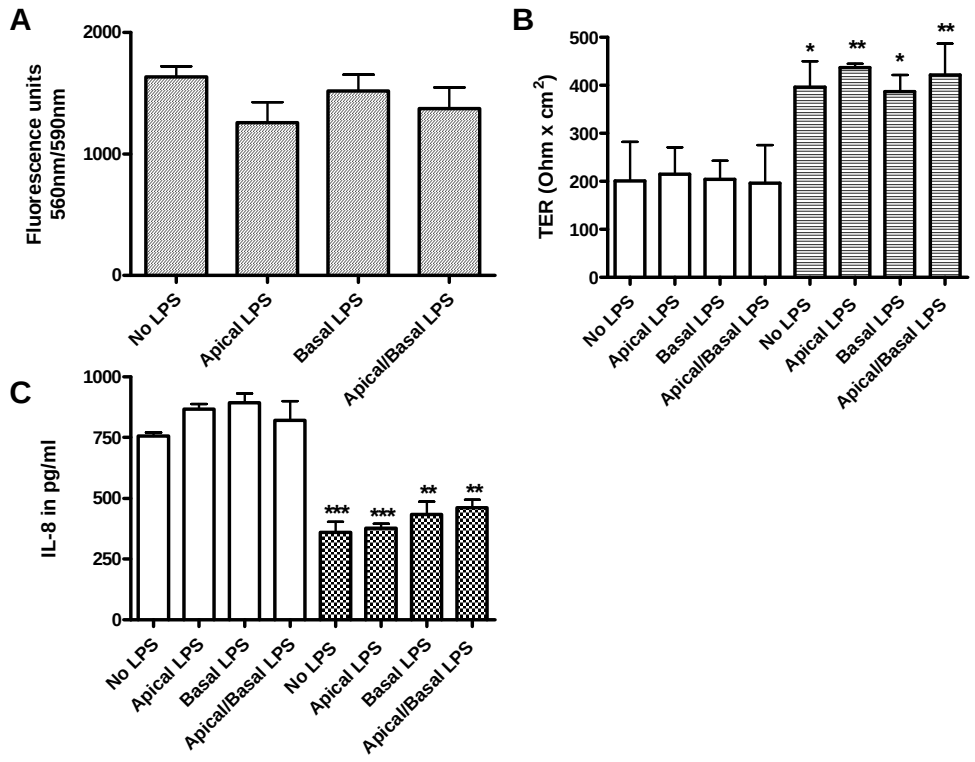

Figure 6.22 Cell viability (A), TER (B) and IL-8 secretion (C) of Calu-3 at ALI after exposure to *B. cepacia* LPS Calu-3 were grown on TWs for 14 days before these were challenged with 1000ng/ml *B. cepacia* LPS, which was applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs, which were only exposed to growth medium. Cell viability of Calu-3 at ALI was not affected by the presence of *B. cepacia* LPS for 24 h. The measured TER, however, was significantly higher after this incubation in all cases including the control. IL-8 secretion was not changed comparing apical supernatants (clear bars) to basolateral media samples (checked bars) but was in all cases significantly lower when measured in the basolateral samples compared to their corresponding apical sample. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3 mono cultures were challenged apically, basolaterally and simultaneously on both sides with 1000 ng/ml *B. cepacia* LPS. The cell viability (figure 6.22 A) of these mono-cultures was not affected by the exposure to this concentration of *B. cepacia* LPS in either way after 24 h incubation. The baseline fluorescence that was measured for the control was 1633.76 ± 152.16 FU. The electrical resistance (figure 6.22 B) was measured before (clear bars) and straight after (striped bars) the incubation with LPS. TER was significantly increased in all cases, including the control, which had a TER of 200.57 ± 81.59 Ω x cm^2 beforehand and a TER of 396.15 ± 53.74 Ω x cm^2 after the 24 h challenge period. Apically challenged TWs had a TER of 215.19 ± 56.01 before the exposure and 436.52 ± 8.66 Ω x cm^2 after the challenge. When treated from the basolateral

side the TER after the challenge was 387.02 ± 34.35 Ω x cm^2 compared to 204.82 ± 37.89 Ω x cm^2 beforehand. Calu-3 mono-cultures were also treated simultaneously from both sides and the resulting TER was 421.34 ± 66.09 Ω x cm^2, whereas it was 196.06 ± 79.42 Ω x cm^2 before the challenge. The measured IL-8 in apical supernatants (clear bars) or basolateral collected medium samples (checked bars) were not increased compared to the control (figure 6.22 C). However, IL-8 concentrations were observed to be significantly lower in all basolateral medium samples compared to the corresponding apical supernatant. This was also found for the control TWs, where the IL-8 concentration was 756.30 ± 23.81 pg/ml in the apical supernatant and 359.58 ± 76.56 pg/ml in the basolateral medium.

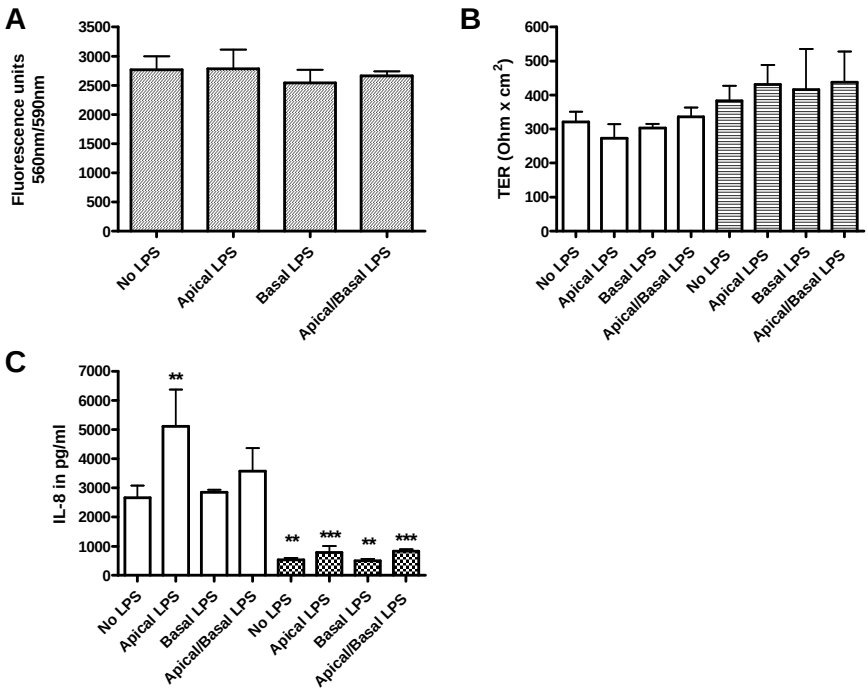

Figure 6.23 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 mono-cultures after exposure to LPS of *P. aeruginosa* 10

Calu-3 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa* 10 LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A) and TER (B) and were examined and all found to be unchanged compared to applicable controls throughout this experiment. IL-8 concentration (C), however, showed a significant increase in apical supernatants (clear bars) when challenged apically and again all basolateral medium samples have significantly lower IL-8 concentrations compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3 mono-cultures were challenged with *P. aeruginosa* 10 LPS (1000 ng/ml) after these were grown at ALI for 14 days. LPS was applied in three different ways, which were apically,

basolaterally or simultaneously from both sides. Cell viability (figure 2.23 A) was analysed straight after 24 h incubation with this type of LPS and was observed not to be influenced, when compared to the baseline fluorescence of the control, which was 2762.33 ± 233.38 FU. Before LPS was added to target compartments of TWs with Calu-3 mono-cultures, the TER (figure 6.23 B) of each TW was measured. After 24 h exposure to *P. aeruginosa* 10 LPS, no significant differences could be identified. TER of the control measured, was 321.31 ± 29.54 Ω x cm^2 beforehand and it was 382.76 ± 44.99 Ω x cm^2 after the challenge. IL-8 secretion (figure 6.23 C) was only significantly increased in the apical supernatant after LPS was added apically. The control TWs had an IL-8 concentration of 2657.94 ± 413.77 pg/ml compared to 5109.82 ± 1264.78 pg/ml after the apical challenge in the apical supernatants (clear bars). This significant difference was not observed in the basolateral medium sample (checked bar) following the apical challenge. However, all basolateral samples had a significantly lower concentration of IL-8 compared to their corresponding apical sample.

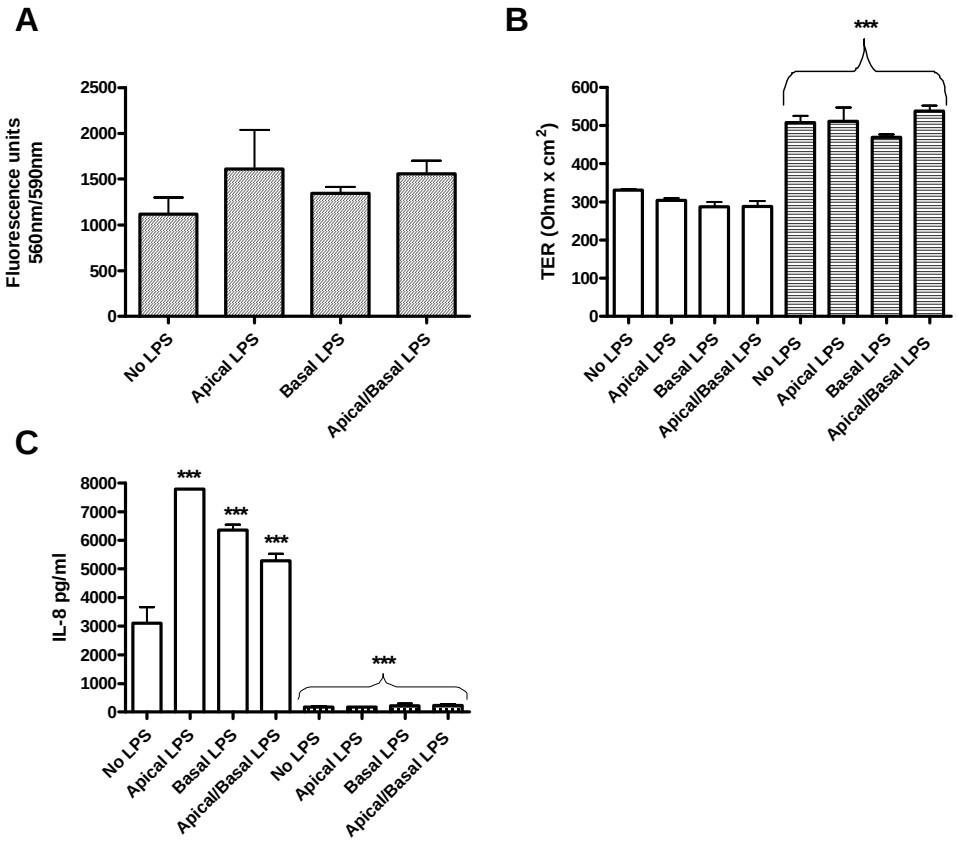

Figure 6.24 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 mono-cultures after exposure to LPS of *P. aeruginosa* 50DR

Calu-3 mono-cultures were grown at ALI for 14 days before they were exposed to *P. aeruginosa* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were exposed to medium only. Cell viability (A) did not show any significant differences after 24 h incubation, when compared to control. TER (B) measured before (clear bars) and after (striped bars) the experiment was found to be significantly higher in all TWs, including the control, after the exposure to LPS. IL-8 secretion (C) on the other hand was found to be significantly increased for all three challenges in the apical supernatants (clear bars) compared to the control. IL-8 concentrations in basolateral medium samples showed again significant lower IL-8 concentrations compared to their corresponding apical supernatant for this cell line. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The cell viability (figure 6.24 A) of Calu-3 mono-cultures grown at ALI was not significantly changed by exposure to 1000 ng/ml of *P. aeruginosa* 50DR LPS for 24 h (baseline fluorescence of control = 1117.50 ± 180.58 FU). The control TWs were exposed to medium only, whereas the LPS was applied either apically, basolaterally or simultaneously from both sides. TER (figure 6.24 B) of these cultures was measured before (clear bars) and after (striped bars) these challenges and interestingly all TWs, including the control, showed a significant increase in electrical resistance after 24 h exposure compared to their corresponding value beforehand. In order, the control increased from 331.026 ± 2.66 Ω x cm^2 to 507.39 ± 18.7 Ω x cm^2, apically challenged TWs showed an increase from 303.82 ± 5.72 Ω x cm^2 to 511.61 ± 35.79 Ω x cm^2, basolaterally challenged ones

increased from 287.467 ± 12.97 Ω x cm^2 to 469.40 ± 7.3 Ω x cm^2 and TER of simultaneous challenged Calu-3 went from 287.72 ± 14.64 Ω x cm^2 up to 538.23 ± 14.56 Ω x cm^2. IL-8 secretion (figure 6.24 C) was measured in apical supernatants (clear bars) as well as in the basolateral medium (checked bars). The apical supernatant of the control had an IL-8 concentration of 3106.57 ± 561.96 pg/ml and only 176.09 ± 36.68 pg/ml were found in the basolateral medium. When Calu-3 were apically challenged, 7790.17 pg/ml of IL-8 was found in the apical supernatant and 177.08 ± 9.18 pg/ml were found in the basolateral medium. For the basal and simultaneous challenges from both sides these findings were 6361.06 ± 189.27 pg/ml and 5288.41 ± 238.77 pg/ml in apical supernatants and 227.54 ± 66.84 pg/ml and 234.75 ± 42.64 pg/ml in the basal medium samples. All basolateral samples were also significantly lower in IL-8 compared to their corresponding apical supernatant.

6.4.3 Cell viability and IL-8 release of co-cultures at ALI after exposure to LPS

6.4.3.1 Exposure of HPF-C38 co-cultures at ALI to LPS

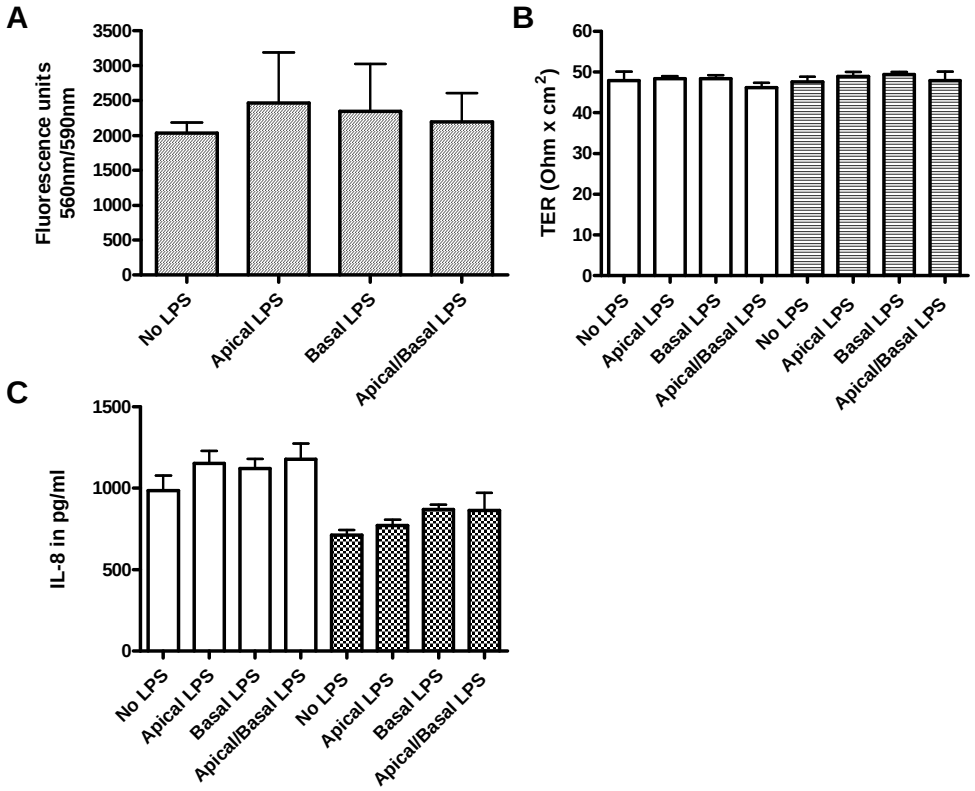

Figure 6.25 Cell viability (A), TER (B) and IL-8 release (C) after exposure to *B. cepacia* LPS of HPF-C38 co-culture grown at ALI
HPF-C38 co-culture was challenged with *B. cepacia* LPS, which was applied to either the apical side of the culture, to the basolateral side or to both at the same time. HPF-C38 on their own without bacteria were treated with medium only and served as control. In this case cell viability, TER and IL-8 secretion was not affected by *B. cepacia* LPS. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-C38 co-cultures were grown at ALI for 14 days before they were challenged with *B. cepacia* LPS, which was applied either apically, basolaterally or simultaneously from both sides at a concentration of 1000ng/ml apart from the control TWs, which were exposed to SF-growth medium only. After 24 h incubation, cell viability (figure 6.25 A) was analysed and no significant changes were seen, when compared to the measured fluorescence baseline of the control, which was 2033.24 ± 265.78 FU. Before the challenge TER (figure 6.25 B) of these co-culture were measured as well as afterwards (striped bars) but this was also observed to be unaffected by this type of LPS. For the control TWs the TER was 47.89 ± 2.24 Ω x cm^2 before the challenge and it was 47.56 ± 1.21 Ω x cm^2 after the challenge. All apical supernatants (clear bars) and all

basolateral medium samples (checked bars) were collected and assayed for the concentrations of IL-8 (figure 6.25 C). In the control there was 987.0 ± 160.8 pg/ml in the apical supernatant and 714.97 ± 50.12 pg/ml in the basolateral medium. All samples analysed had similar concentrations to these.

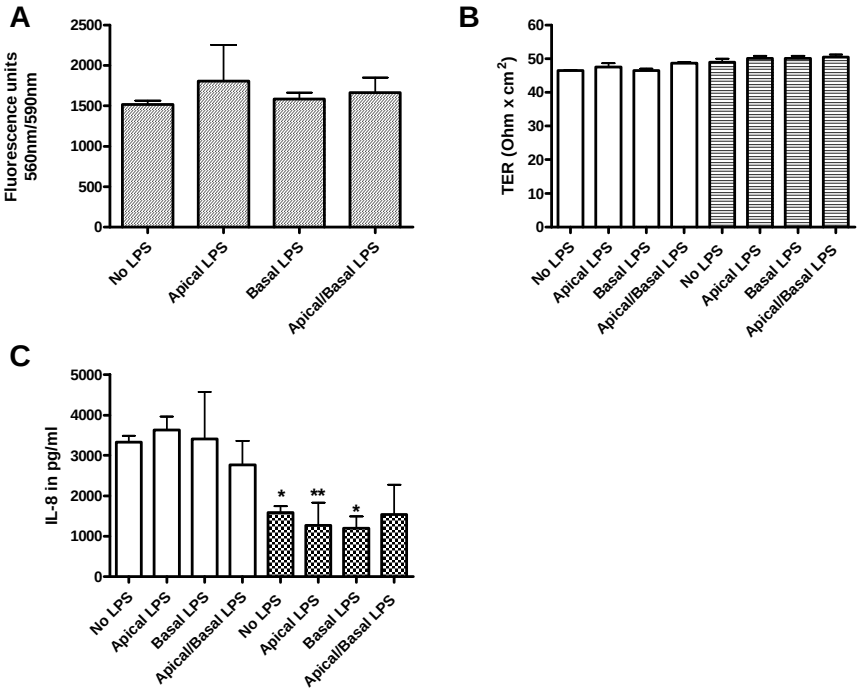

Figure 6.26 Cell viability (A) TER (B) and IL-8 release (C) of HPF-C38 co-culture after exposure to *P. aeruginosa* 10 LPS
HPF-C38 co-cultures were grown at ALI on TWs for 14 days before these cells were exposed to *P. aeruginosa* 10 LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability and TER measurements were not affected by exposure to this type of LPS after 24 h exposure. IL-8 release of HPF-C38 co-culture was not induced in either apical supernatants (clear bars) or in basolateral medium samples (checked bars). However, basolateral medium was significantly lower in its IL-8 concentration, when apically challenged or from both sides simultaneously compared to the corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-C38 co-cultures were challenged with *P. aeruginosa* 10 LPS after they were grown at ALI for 14 days. Control TWs were exposed to medium only whereas the other TWs were challenged either apically, basolaterally or simultaneously with 1000 ng/ml LPS of *P. aeruginosa* 10. The cell viability (figure 6.26 A) was checked after the 24 h incubation with LPS and was unaffected by this event compared to the control (baseline fluorescence = 1517.48 ± 48.13 FU). TER (figure 6.26 B) was found to be stable throughout the experiment and showed similar values, when

measured before (clear bars) and after (striped bars) the challenges. The control TWs had a TER of 46.53 ± 0.19 Ω x cm^2 beforehand and a TER of 48.99 ± 0.99 Ω x cm^2 after the exposure to LPS. IL-8 secretion (figure 6.26 C) was also assayed and found to be unchanged in apical supernatants (clear bars) as well as in basolateral medium samples (checked bars). IL-8 concentrations found in the apical supernatant of the control was 3322.86 ± 160.33 pg/ml and it was 1582.63 ± 164.90 pg/ml, which was significantly lower in the basolateral medium of the control. Basolateral medium samples of apically challenged and basolaterally challenged were also observed to be significantly lower in IL-8 than their corresponding apical supernatant. Apically challenged HPF-C38 co-cultures had 3628.83 ± 333.41 pg/ml in their apical supernatant and basolaterally challenged ones had 3405.64 ± 1168.02 pg/ml of IL-8. Their corresponding basal medium samples had 1273.94 ± 557.45 pg/ml and 1193.12 ± 299.56 pg/ml of IL-8.

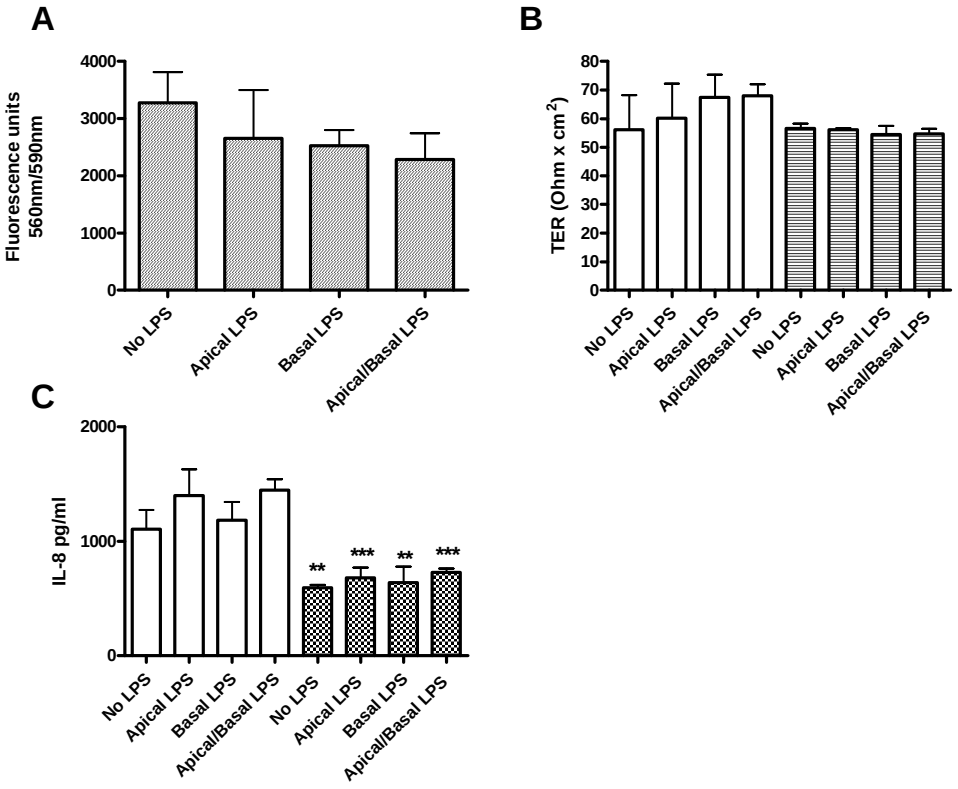

Figure 6.27 Cell viability (A), TER (B) and IL-8 secretion (C) of HPF-C38 grown at ALI after exposure to *P. aeruginosa* 50DR LPS

HPF-C38 were grown on TWs at ALI for 14 days before they were challenged with *P. aeruginosa* LPS, which was applied either apically, basolaterally or from both sides simultaneously. HPF-C38's cell viability and measured TER before (clear bars) and after the challenges (striped bars) were not changed by this 24 h incubation with *P. aeruginosa* LPS. Neither was the IL-8 secretion of this co-culture significantly increased in apical supernatants (clear bars) or basolateral medium samples (checked bars). However, basolateral samples were all, including the control, significantly lower in IL-8 concentrations compared to their corresponding apical supernatants. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

After HPF-C38 were challenged with *P. aeruginosa* 50DR LPS, the cell viability (figure 6.27 A) was analysed. Compared to the control, of which the baseline fluorescence was 3276.43 ± 538.73 FU, none of the challenges influenced the cells cell viability, whether LPS was applied apically, basolaterally or simultaneously from both sides. TER (figure 6.27 B) was monitored before (clear bars) and after (striped bars) each challenge and was observed to be stable throughout the experiment. All readings were similar to the control, which had a TER of 56.11 ± 12.11 Ω x cm^2 beforehand and 56.61 ± 1.60 Ω x cm^2 afterwards. IL-8 secretion (figure 6.27 C) was not induced by 1000 ng/ml LPS of *P. aeruginosa* applied in the different compartments of the TWs. In all cases, including the control, were basolateral IL-8 concentrations (checked bars) significantly lower compared to their corresponding apical supernatants (clear bars). In the control these concentrations were 1107.21 ± 166.67 pg/ml on the apical side and 594.59 ± 26.43 pg/ml on the basal side of the co-culture.

6.4.3.2 Exposure of HPF-IB3-1 co-cultures to LPS

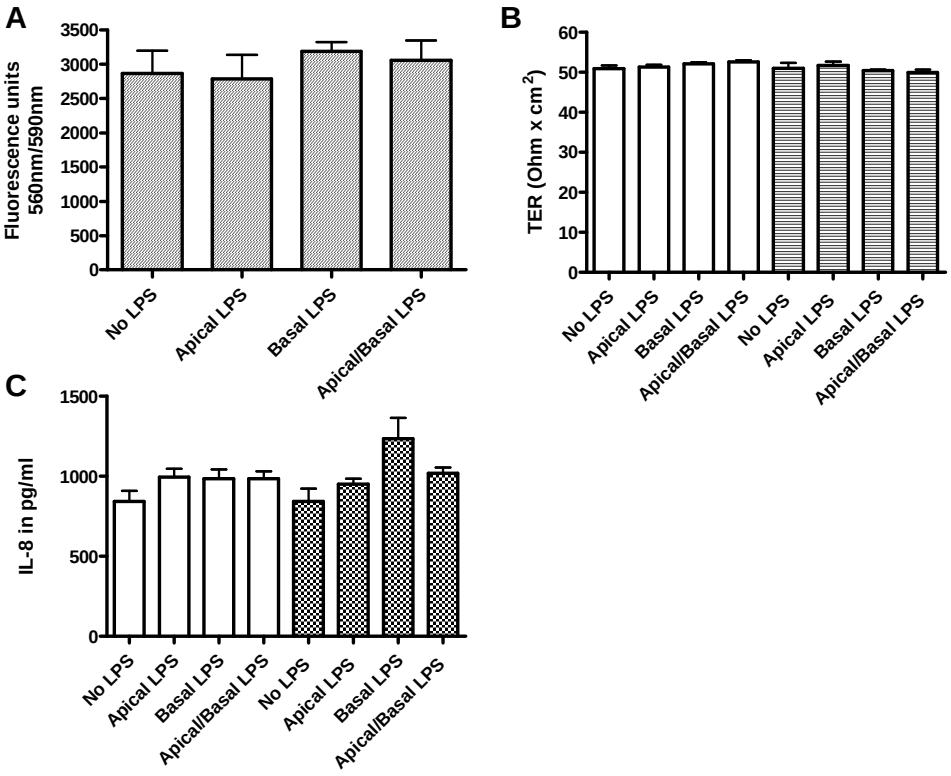

Figure 6.28 Cell viability (A), TER (B) and IL-8 release (C) after exposure to *B. cepacia* LPS of HPF-IB3-1 grown at ALI
HPF-IB3-1 co-cultures were at ALI for 14 days before they were challenged with *B. cepacia* LPS, which was applied either apically, basolaterally or from both sides simultaneously. HPF-IB3-1 control TWs were challenged with medium only. In this case neither cell viability, TER nor IL-secretion was significantly affected by any of these three challenges with *B. cepacia* LPS for 24 h. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1 were also challenged with *B. cepacia* LPS (1000ng/ml) for 24 h, which was applied in three different ways. TWs were either challenged from the apical side, the basal side or from both sides at the same time. LPS had no significant affects on HPF-IB3-1's cell viability (figure 6.28 A), where the control had a baseline fluorescence of 2866.65 ± 578.51 FU. TER (figure 6.28 B), which was measured before (clear bars) and after (striped bras) the challenges was observed to be stable throughout the experiment and all results were similar to the control, which had a TER of 50.85 ± 0.80 Ω x cm^2 beforehand and a TER of 51.04 ± 1.32 Ω x cm^2 afterwards. IL-8 secretion (figure 6.28 C) was not increased by the exposure to *B. cepacia* LPS and concentrations found in the control were 844.99 ± 110.49 pg/ml on the apical side (clear bars) and 843.40 ± 138.15 pg/ml on the basolateral side (checked bars).

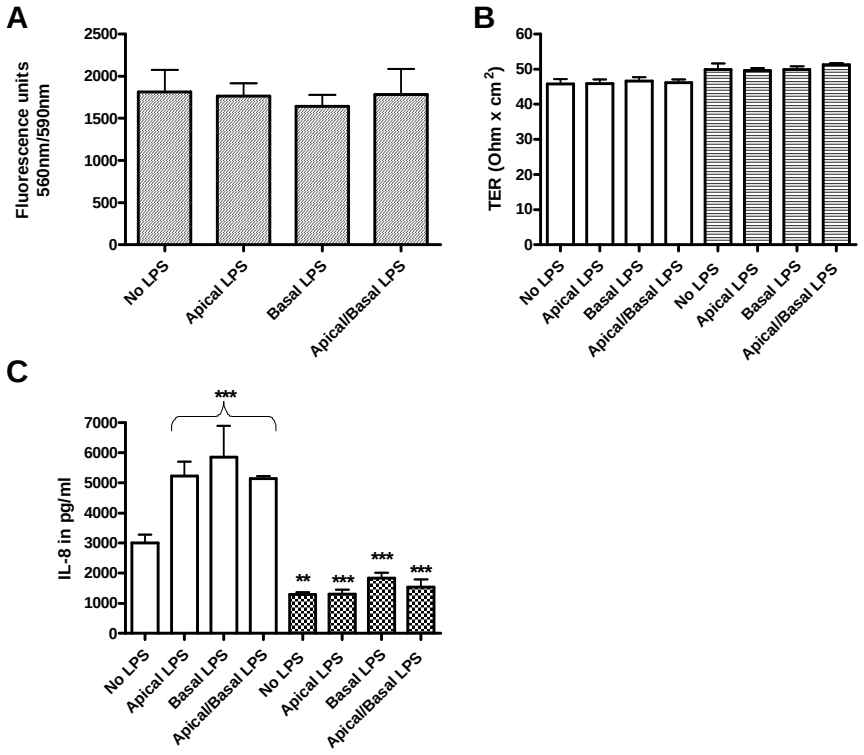

Figure 6.29 Cell viability (A) TER (B) and IL-8 release (C) of HPF-IB3-1 co-culture after exposure to *P. aeruginosa* 10 LPS
HPF-IB3-1 co-cultures were on TWs for 14 days before these cells were exposed to *P. aeruginosa* 10 LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability and TER measurements were not affected by exposure to this type of LPS after 24 h exposure. IL-8 secretion was induced by apically, basolaterally treatment with LPS of *P. aeruginosa* 10, as well as by a simultaneous treatment of HPF-C38 in apical supernatants (clear bars). All basolateral collected samples (checked bars) showed a significantly lower IL-8 concentration, when compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1 co-cultures were challenged with *P. aeruginosa* 10 LPS after they were grown at ALI for 14 days. Control TWs were exposed to medium only whereas the other TWs were challenged either apically, basolaterally or simultaneously with 1000 ng/ml LPS of *P. aeruginosa* 10. The cell viability (figure 6.29 A) was checked after the 24 h incubation with LPS and was unaffected by this event compared to the control (baseline fluorescence = 1517.48 ± 48.13 FU). TER (figure 6.29 B) of this co-culture was stable throughout the experiment and showed similar values before (clear bars) and after (striped bars) the challenges. TER of the control was 45.76 ± 1.44 Ω x cm^2 before the challenge and it was 49.90 ± 1.65 Ω x cm^2 after the challenge. IL-8 secretion (figure 6.29 C), however, was significantly increased by all three different exposures. In the apical supernatant (clear bars) of control TWs the mean IL-8 concentration found was 3006.32 ± 273.16 pg/ml compared to 5228.74 ± 466.28 pg/ml after the apical challenge, compared to 5855.73 ± 1038.51 pg/ml after the basolateral challenge and compared to 5140.15 ± 75.73 pg/ml after the

simultaneous challenge from both sides. In the basolateral medium samples, which were all significantly lower compared to their apical samples, although LPS did not alter basal IL-8 compared to control. The corresponding values were 1294.59 ± 79.11 pg/ml for the control, 1307.7 ± 147.41 pg/ml for the apical challenge, 1827.48 ± 188.61 pg/ml for the basolateral challenge and 1540.59 ± 245.4 pg/ml for the simultaneous challenge from both sides.

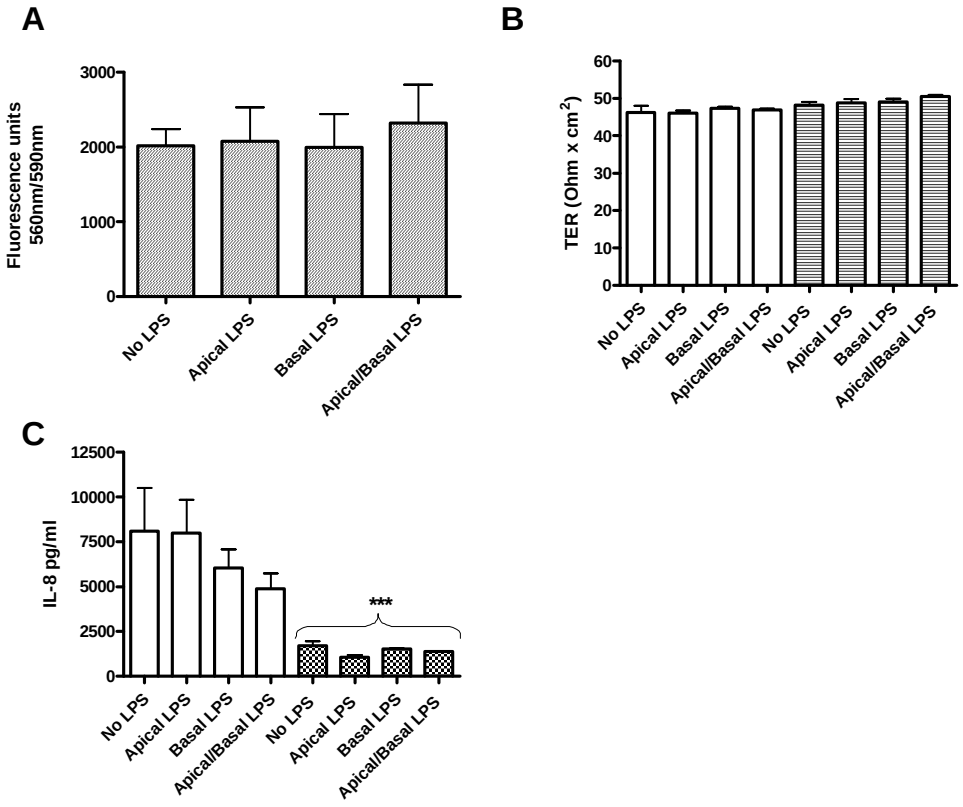

Figure 6.30 Cell viability (A), TER (B) and IL-8 secretion (C) of HPF-IB3-1 grown at ALI after exposure to *P. aeruginosa* 50DR LPS
HPF-IB3-1 were grown at ALI for 14 days before they were induced with *P. aeruginosa* LPS, which was applied either apically, basolaterally or from both sides simultaneously. Neither HPF-IB3-1's cell viability nor the TER measured after the challenges (striped bars) were affected by this 24 h incubation with *P. aeruginosa* LPS. IL-8 secretion did not significantly change in apical supernatants (clear bars) or basolateral medium samples (checked bars) but again the basolateral samples were all, including the control, significantly lower in IL-8 concentrations compared to their corresponding apical supernatants. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1 co-cultures were exposed to *P. aeruginosa* 50DR LPS at a concentration of 1000 ng/ml. LPS was either applied apically, basolaterally or from both sides at the same time, apart from control TWs, which were exposed to SF-medium only. Analysis of cell viability (figure 6.30 A) revealed that LPS has no affect on the co-cultures viability, where the baseline fluorescence of

the control was 2015.08 ± 227.20 FU. TER (figure 6.30 B) was again found to be stable throughout the experiment and all mean TERs were comparable to the control, which had a TER of 46.31 ± 1.71 Ω x cm^2 before the challenge and a TER of 48.29 ± 0.76 Ω x cm^2 afterwards. No induction of IL-8 secretion (figure 6.30 C) was noted for any of the challenges, when compared to control, which had an IL-8 concentration of 8101.58 ± 2402.56 pg/ml in the apical supernatant and 1716.73 ± 250.67 pg/ml in the basolateral medium. All basolateral samples showed a significantly lower IL-8 concentration after all challenges, when compared to their corresponding apical supernatants.

6.4.3.3 Exposure of HPF-Calu-3 co-cultures to LPS

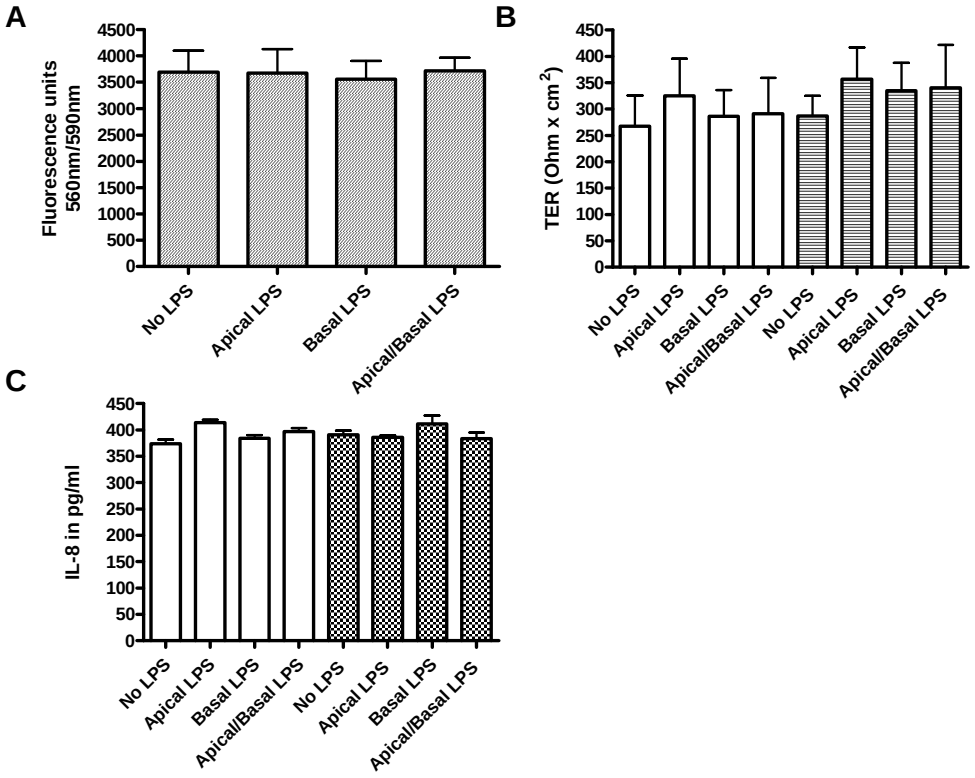

Figure 6.31 Cell viability (A), TER (B) and IL-8 release (C) after exposure to *B. cepacia* LPS of HPF-Calu-3 co-culture grown at ALI
HPF-Calu-3 co-culture was challenged with *B. cepacia* LPS, which was applied to either the apical side of the culture, to the basolateral side or to both at the same time. HPF-C38 on their own without bacteria were treated with medium only and served as control. In this case cell viability, TER and IL-8 secretion was not affected by *B. cepacia* LPS. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-Calu-3 co-cultures were exposed to *B. cepacia* LPS for 24 h with LPS added to the SF-culture medium either in the apical compartment, the basolateral compartment or in both compartments at the same time. This co-culture's cell viability (figure 6.31 A) was not affected by this type of LPS as all measured fluorescence signals were similar to the control, of which the baseline fluorescence was 3695.40 ± 704.20 FU. Neither did the TER (figure 6.31 B) show a significant change over this 24 h incubation. The control TWs had a TER of 267.67 ± 57.92 Ω x cm^2 before (clear bars) the exposure to LPS and a TER of 287.17 ± 37.86 Ω x cm^2 afterwards (striped bars). IL-8 secretion (figure 6.31 C) was similar in all apical samples as well as in all basolateral samples analysed, including the control TWs, which had an IL-8 concentration of 374.04 ± 12.76 pg/ml in the apical supernatant (clear bars) and 390.54 ± 14.83 pg/ml in the basolateral medium (checked bars).

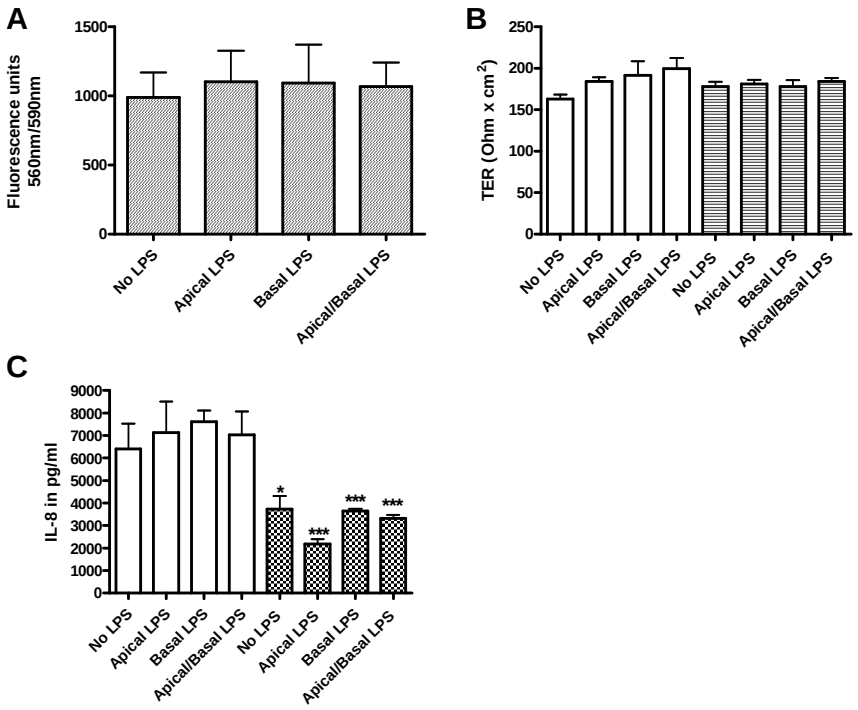

Figure 6.32 Cell viability (A) TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to *P. aeruginosa* 10 LPS

HPF-Calu-3 co-cultures were grown at ALI on TWs for 14 days before exposure to *P. aeruginosa* 10 LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability and TER measurements were not affected by a 24 h exposure to this type of LPS. IL-8 secretion was not induced by any of the three treatments with LPS of *P. aeruginosa* 10. All basolateral collected samples (checked bars) showed a significantly lower IL-8 concentration, when compared to their corresponding apical supernatant (clear bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-Calu-3 co-cultures at ALI were challenged with *P. aeruginosa* 10 LPS. Control TWs were only exposed to SF-culture medium, whereas the other TWs were challenged in three different ways. LPS was added either apically, basolaterally or simultaneously at the same time. Afterwards cell viability (figure 6.32 A) was analysed and found to be unchanged, when comparing results of challenged TWs to the control, of which the baseline fluorescence was 989.14 ± 178.58 FU. TER (figure 6.32 B) was also monitored before (clear bars) and after (striped bars) the challenges but no significant differences were observed. Before the exposure to this type of LPS the TER of the control was 162.84 ± 5.13 Ω x cm^2 and it was 178.09 ± 5.51 Ω x cm^2 after the challenge. IL-8 secretion (figure 6.32 C) was not induced by LPS, no matter which way it was applied. Significantly differences were only observed for basolateral samples, which were all lower in IL-8 compared to their corresponding apical supernatants. Concentrations of IL-8 in the apical supernatant (clear bars) were all similar to the control, in which it was 6401.12 ± 1131.92 pg/ml. In the basal medium the same was observed with the control containing 3726.11 ± 583.77 pg/ml of IL-8.

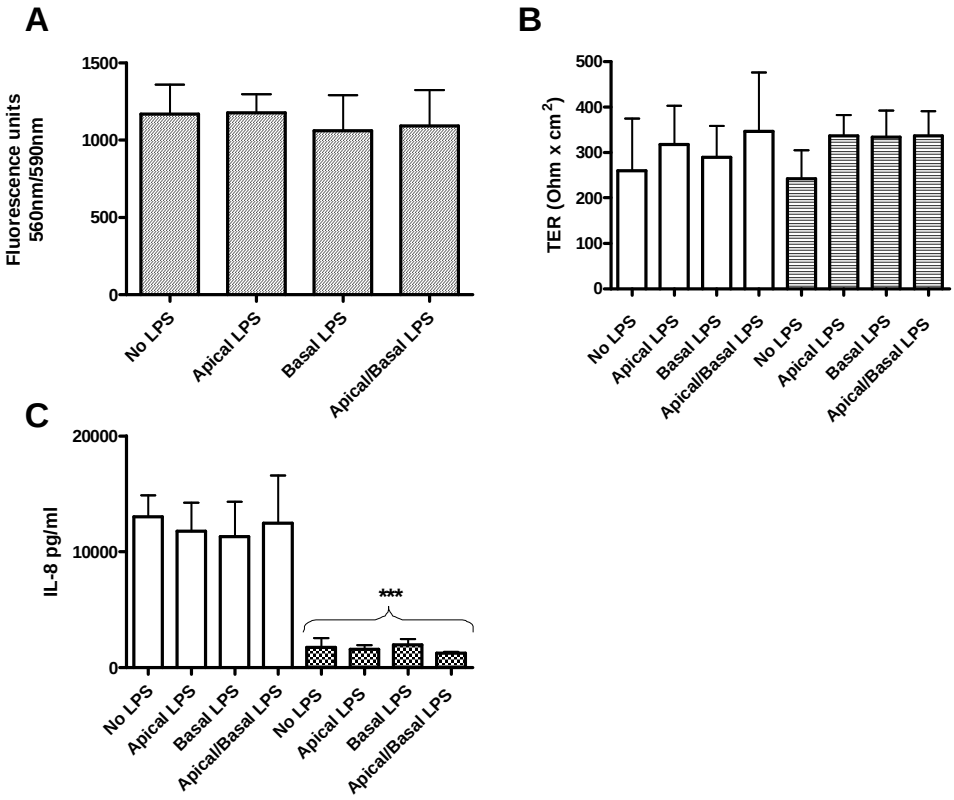

Figure 6.33 Cell viability (A) TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to *P. aeruginosa* 50DR LPS
HPF-Calu-3 co-cultures were grown at ALI on TWs for 14 days before exposure to *P. aeruginosa* 50DR LPS, which was applied either apically, basally or simultaneously from both sides. Cell viability was not affected after 24 h incubation compared to the control TWs and neither was TER affected by a 24 h exposure to this type of LPS. IL-8 secretion was not induced by any of the three treatments with LPS of *P. aeruginosa*. All basolateral collected samples (checked bars) showed a significantly lower IL-8 concentration, when compared to their corresponding apical supernatant (clear bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-Calu-3 were exposed to *P. aeruginosa* 50DR LPS after they were grown at ALI for 14 days. LPS was applied to the apical side, to the basal side or to both sides at the same time and cell viability (figure 6.33 A) was assayed straight after the 24 h challenge and was observed to be unaffected, compared to the control's baseline fluorescence of 1171.24 ± 187.91 FU. TER (figure 6.33 B) was also unchanged by these challenges and all TWs showed similar readings before (clear bars) and after (striped bars) the LPS exposure like the control. TER of the control was 259.86 ± 115.42 Ω x cm^2 before the challenge and it was 242.88 ± 62.30 Ω x cm^2 after the challenge. IL-8 concentrations (figure 6.33 C) were assayed as well and no differences were found in the apical supernatants (clear bars) or in the basolateral medium samples (checked bars). The IL-8 concentration observed in the apical supernatant of the control was 13006.16 ± 1858.64 pg/ml and it was 1769.39 ± 787.33 pg/ml in the basal medium analysed. Again all

basolateral medium samples have a significantly lower concentration of IL-8 compared to their corresponding apical samples.

6.4.4 TLR-4 and CD14 surface expression

The surface expression of TLR-4 and CD14 was measured by flow cytometry after direct and indirect immunofluorescent staining with anti-TLR-4 and anti-CD14 antibodies (figure 6.34)

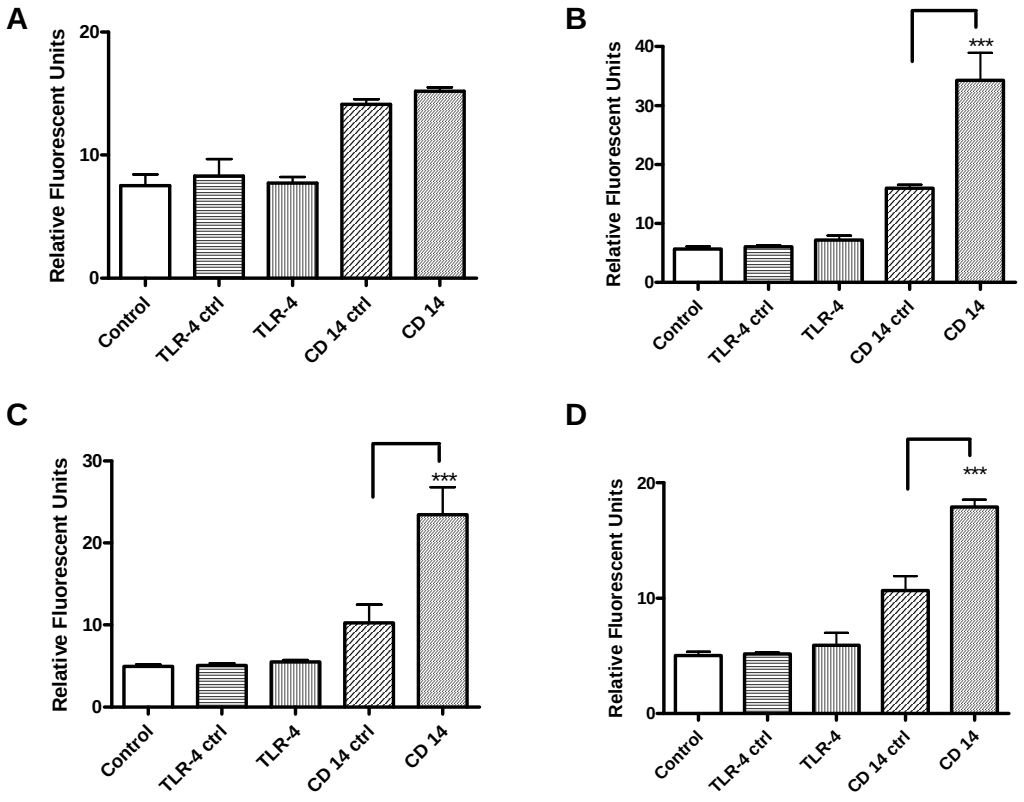

Figure 6.34 TLR4 and CD14 surface expression of HPF (A), C38 (B), IB3-1 (C) and Calu-3 (D)
Using direct and indirect immunoflorescent antibody staining (anti-TLR-4 and anti-CD14) on submerged unstimulated cells in connection with flow cytometry showed the surface membrane expression of these receptors on each cell type used. None of the cells analysed had TLR-4 expressed on the cell surface membrane. Neither did HPF show CD14 surface expression but all three epithelial cell lines showed CD14 expressed on the surface as analysed by the significant increases of relative fluorescent units after staining with anti-CD14 and compared to the control antibody. (n=3)

HPF did not show any membrane surface expression of TLR-4 or CD14. The fluorescent signal for the specific antibodies compared to the control antibodies was not observed to be significantly increased. However, for all three epithelial cell lines there was a significant increase in

fluorescent signal after staining these cells with anti-CD14 antibody and compared to the control antibody's fluorescent signal. No TLR-4 expression was found on the epithelial cells though.

6.5 Discussion

In this chapter submerged mono-cultures as well as mono- and co-cultures at ALI were challenged with three different types of LPS. One was a commercially available LPS, which is isolated from *P. aeruginosa* serotype 10 (Sigma) and the other two were phenol extracted previous to these experiments (see Chapter 2.13). One was extracted from a *P. aeruginosa* strain called 50DR, which is a CF isolate and the other one was extracted from *B. cepacia* J2315. Cellular responses were analysed in terms of cell viability, cell layer integrity when cultured on TWs and the pro-inflammatory cytokine secretion of IL-8 was observed. In addition, the expression of the surface membrane receptor expression required to respond to LPS stimulation, was analysed by direct and indirect immunofluorescence antibody staining followed by flow cytometric analyses.

6.5.1 HPF mono-cultures challenged with LPS

HPF mono-cultures either under submerged conditions on 24-well plates or grown under submerged conditions on TWs were not influenced by exposure to LPS looking at their cell viability (figures 6.1-6.3, 6.13-6.15). In terms of IL-8 secretion a significant increase was observed in submerged mono-cultures in response to *B. cepacia* LPS as well as for *P. aeruginosa* 10 LPS. At 1000ng/ml LPS, both types of LPS induced the same strength of inflammatory response in these cells, which is surprising as it has been reported that *B. cepacia* LPS is more potent than *P. aeruginosa* LPS, at least in terms of activating monocytes (Shaw *et al*., 1995, Zughaier *et al*., 1999). Koyama *et al*. (2000) reported that, at a concentration of 100 µg/ml of *P. aeruginosa* LPS, human fetal lung fibroblasts responded positively and released neutrophil chemotactic activity (IL-8 and granulocyte colony-stimulating factor (G-CSF). The group did not show data of IL-8 release for each cell line but the results presented by this group underlines the responsiveness of airway fibroblasts to LPS activation and that these cell types take actively part in pro-inflammatory responses by secreting IL-8 (Koyama *et al*., 2000). This pro-inflammatory response has recently been shown for primary human lung fibroblasts under the same conditions as were used here. After 24 h exposure to 1 µg/ml LPS (*E. coli*) these fibroblasts showed a significant increase in IL-8 secretion (Zhang *et al*., 2011).

When HPF were grown on TWs they only responded to *P. aeruginosa* 10 LPS and IL-8 was found to be 3 times as high in concentration compared to submerged cultures on 24-well plates. Interestingly this was only observed when HPF were challenged simultaneously from both sides. Little increases were seen for the other two challenges but were not found significant. These

results suggest that receptors, such as PRR TLR-4 are expressed by HPF to initiate the pro-inflammatory signalling pathway necessary for IL-8 production.

In figure 6.34 A it is shown that HPF did not have TLR4 or CD14 expressed on their surface membrane when these cells were unstimulated, whether the LPS signalling pathway receptors are intracellular and whether they are translocated to the surface membrane upon stimulation in these cells has to be further analysed. However, it was shown that in corneal fibroblasts TLR-4 expression was increased in a dose-dependent matter, when stimulated with *P. aeruginosa* LPS (Wong *et al*., 2011). Another group studied rat lungs and found that TLR-4 mRNA was not detectable until 6-12h after LPS exposure, which then lasted up to 36h after exposure before the level dropped again (Janardhan *et al*., 2006). Another group looked at human embryonic lung fibroblasts and also reported that TLR-4 mRNA was not detectable by PCR (Paladino *et al*., 2006). All together these data indicate that expression of TLR-4 on lung fibroblasts is initiated upon LPS exposure but further investigation is necessary to confirm TLR-4 surface expression after HPF were stimulated with LPS.

6.5.2 Challenge of submerged epithelial mono-cultures and mono-cultures at ALI with LPS

Epithelial cells are the first line defenders in the airways and encounter different inhaled particles, including bacteria, viruses and LPS, an endotoxin of gram-negative bacteria. It is controversial whether human airway epithelial cells in culture respond to challenge with LPS or not. Another area of controversy that was observed throughout the years of CF research is the debate about, which comes first inflammation based on the CFTR defect and the consequences of it or does infection trigger the highly inflammatory state seen in lungs of CF. There are reports, which support the pre-inflammatory state of CF cell lines (Kube *et al*., 2001) and also the IL-8 concentration in BAL fluid has shown a significant increase in CF compared to healthy patients (Bonfield *et al*., 1995). In contrast other groups have found that there is no difference in IL-8 release in either primary or cell line epithelial cells (John *et al*., 2010, Becker *et al*., 2004). Here we find that IB3-1 secretes slightly higher IL-8 compared to C38 at baseline level but this was not consistent throughout this whole project and due to the large amount of material supporting both types of hypothesis it is hard to currently explain any mechanism behind these observations.

Here we find that under submerged conditions LPS did not stimulate IL-8 secretion in the epithelial cell line C38 and IB3-1, except for two challenges. C38 did respond to *B. cepacia* LPS, when stimulated with 100 ng/ml or 1000 ng/ml but the response was quite low considering that

the baseline secretion was 21.39 ± 1.25 pg/ml and the highest IL-8 concentration after stimulation came only up to 46.5 ± 6.75 pg/ml. Concerning the LPS type it has been reported that *B. cepacia* LPS is a more potent immunomodulatory stimuli compared to LPS from *P. aeruginosa* (Zughaier *et al*., 1999), which is consistent with findings here. However the LPS concentrations used here are quite low compared to the concentrations used by other groups. Palfreyman *et al*. (1997) showed that A549 cells in submerged mono-cultures were not stimulated by 10µg/ml LPS from *B. cepacia* (Palfreyman *et al*., 1997). In contrast, another group has shown that 10µg/ml *P. aeruginosa* 10 LPS is able to induce A549 cells to produce neutrophil chemotactic activity, including IL-8, but measured after prolonged (72 h rather than 24 h) exposure. This group indicated that responsiveness was dependent on the bacterium and serotype of LPS (Koyama *et al*., 2000). Controversially, as already mentioned before it has been reported that *B. cepacia* LPS is a more potent inflammatory stimuli than *P. aeruginosa* LPS (Zughaier *et al*., 1999), which is not reflected in the results of these two studies (Palfreyman *et al*., 1997, Koyama *et al*., 2000).

The difference in IL-8 response between C38 and IB3-1 is in accordance with findings in CF patients. It has been shown that TLR-4 is reduced in the airway epithelium of patients with CF compared to healthy controls (Hauber *et al*., 2005, John *et al*., 2010). The vast amounts of IL-8 concentrations usually found in CF patients have been suggested to be due to the increased TLR-4 expression found on cells located in the submucosa, especially on neutrophils. 60 % of the TLR-4 positive cells in CF lungs are neutrophils and the overall number of these immune cells is massively increased in CF lungs, when compared to normal lungs. On top of these findings it was also reported that TLR-4 positive monocytes/macrophages were increased in numbers in CF compared to non-CF patients (Hauber *et al*., 2005). These findings all together suggest that CF airway epithelial cells might not be able to respond as efficiently to LPS, which could favour bacterial colonisation but considering the other immune cells *in vivo* showing increased TLR-4 expression and knowing that there is a massive neutrophillic infiltration will still lead to constantly increased IL-8 concentrations in CF. In addition it should be kept in mind that live bacteria as found in CF airways display other virulence factors that are also able to induce IL-8 secretion.

Furthermore it has been shown that intracellular TLR-4 has been reported to be located in the Golgi apparatus and there it co-localizes with internalized LPS in intestinal epithelial cells for example (Hornef *et al*., 2002). This could be a way of LPS stimulation in epithelial cells as well, which might be to control inflammatory responses, so that low concentrations of LPS do not evoke a response but high concentrations of free LPS or exposure to life bacteria will. However it

is not known yet whether TLR-4 is relocated to the surface membrane through activation of LPS or through IL-8 or bioactive lipids for example (Guillot *et al*., 2004).

In A549 and BEAS 2B, for example, it has been shown as well that TLR-4 is not expressed on the surface membrane but has been indentified to be present intracellularly in the cytoplasm, whereas in U-937 (monocytic cell line) it has been shown to be present in both membrane and cytoplasm, which underlines the levels of responsiveness of these cells compared to the epithelial ones. It has been shown that phagocytic cells only need to encounter about 1-10 ng/ml of LPS in contrast to epithelial cells that need high concentrations of around 0.1-1 µg/ml for induction of IL-8 secretion (Guillot *et al*., 2004). These concentrations were exactly those C38 responded to.

Membrane surface CD14 expression is significantly higher in C38 (figure 6.34 B) but as CD14 does not have an intracellular signalling domain (that is why it works together with TLR-4/MD-2) this cannot be the reason for such a difference in IL-8 secretion (Kim *et al*., 2005a). It has been reported though that in non-CF lung tissue TLR-4 was found strongly expressed on the apical side of the membrane of epithelial cells, whereas in CF lung tissue there was almost no staining for TLR-4 (John *et al*., 2010). Together with the surface expression of CD14 found here in C38 this could indicate that after stimulation, TLR-4 is relocated to the membrane of C38 and leads to IL-8 secretion via NF-κB, which is partially impaired in IB3-1. As already mentioned above this could lead to an impairment of this one innate immunity pathway and might lead to initial colonisation with bacteria. Additionally the input of other immune cells in CF in terms of TLR-4 mediated signalling after exposure to LPS is huge and still leads to the clinical picture seen in CF with high IL8 concentrations and constant neutrophillic infiltration compared to normal lungs.

When these two cell lines were cultured at ALI though, no IL-8 response was observed at all. At ALI a concentration of 1000ng/ml of LPS of each type was used to stimulate the cells, which might simply not be high enough for resistant airway epithelial cells, when differentiated. As TLR-4 surface expression and CD14 surface was only analysed in unstimulated submerged monolayers of these cells it is impossible to comment on whether it is higher or lower in CF compared to non-CF under these conditions and also whether it is found on the surface membrane or intracellularly. As earlier mentioned in lung tissue of CF and non-CF patients there was a huge difference observed in TLR-4 expression on the apical side of epithelial cells, with non-CF showing strong expression and CF showing reduced expression of TLR-4 (John *et al*., 2010, Hauber *et al*., 2005). John *et al*. (2010) have also reported that similar results for submerged CF and non-CF cell lines. A bronchial epithelial cell line, CFBE (CF) expressed the lowest level of surface TLR-4, when compared to its corrected counterpart corrCFBE and the

plasmid controlled cell line dfCFBE (John *et al*., 2010). For cells grown at ALI analysis methods, such as flow cytometry, cannot be employed and therefore the PRR expression on well-differentiated airway epithelial cells has not been analysed extensively.

TLR-4 and CD14 mRNA studies and immunohistochemistry paraffin sections of these cultures will help to elucidate and compare findings. As C38 is the corrected form of IB3-1 and has been exposed to CF environment before it will be interesting to investigate whether the correction in CFTR function also brings these differences back to a non-CF state, in terms of receptor expression, for example.

Interestingly, when IB3-1 mono-cultures at ALI were stimulated with LPS from the basal side and from apical and basal side at the same time, a decrease in cell viability was observed. Therefore LPS of the CF isolated *P. aeruginosa* strain seems to have a cytotoxic affect on IB3-1 only when these were challenged from the basolateral side.

For whole intact *P. aeruginosa* it has been reported before that on well differentiated epithelial cells binding occurs preferably at the basolateral membrane rather than apically. Furthermore it has been shown that cells at ALI are more susceptibe when encountering P . aeruginosa to its cytotoxic effects (Fleiszig *et al*., 1997). There is definitely evidence that polarized cells react differently upon bacterial challenges compared to submerged cells but whether there is an basolateral expression difference of receptors between these CF and non-CF cells used here needs to be further analysed.

6.5.3 Challenge of co-cultures at ALI with LPS

In the case of HPF-C38 and HPF-IB3-1 there were no significant changes in cell viability or TER observed following challenge in any compartment with any of the LPS tested.

Even though *B. cepacia* LPS was reported to be more potent in terms of causing an inflammatory response compared to *P. aeruginosa* (Zughaier *et al*., 1999), no IL-8 response was observed here for HPF-C38 and HPF-IB3-1. The only IL-8 increase was observed for HPF-IB3-1 after the 24 h challenge with *P. aeruginosa* 10 LPS for all three challenges.

One reason for this difference in response of HPF-IB3-1 to the commercial LPS and the isolated one could be possible differences in the lipid A structure. It has been shown before that isolated *P. aeruginosa* strains from CF patients show different lipid A structures compared to the parental strain or compared to an earlier isolate of the same bacterium from the same patient (Ernst *et al*., 2007, Cigana *et al*., 2009). In general it is thought that the selective pressure in the CF lung leads to modifications of Lipid A, the immunomodulatory component of LPS and Cigana *et al*. (2009) showed a decrease of TLR-4 mediated IL-8 secretion comparing an early LPS isolate

stimulation to a late isolate. It has been shown that bacterial isolates taken later in the course of CF lung disease activate TLR-4 to a lesser extent compared to early isolates of infection from the same patient. This was shown using submerged mono-layers of IB3-1 and C38, where LPS of an early *P. aeruginosa* isolate induced higher IL-8 secretion in both cell lines compared to LPS of late isolates (Cigana *et al*., 2009). However, further studies are required to understand the molecular mechanisms better and the microbial LPS modifications and how exactly this leads to persistent *P. aeruginosa* infections in CF.

The commercial LPS of *P. aeruginosa* serotype 10 did induce IL-8 secretion in HPF-IB3-1 co-cultures but the LPS from the clinical isolate (50DR) did not. No additional information about the patient or the isolate is available and therefore, this makes it difficult to make definite statements here as to whether changes in lipid A over time of infection is the reason for not stimulating these cultures of HPF-IB3-1. Plus these studies presented here were done on well differentiated ALI cultures, where the receptor repertoire on the cell surface and intracellularly is most likely to be different compared to submerged cultures (Fleiszig *et al*., 1997) and has yet to be characterised.

Furthermore it is interesting to note that submerged C38 mono-cultures are responsive to *B. cepacia* LPS but not when grown at ALI or in co-culture with HPF. In contrast IB3-1 do not respond to any LPS when submerged or at ALI but they do when co-cultured with HPF. These results point out that culturing cells at ALI can cause a different immune response of epithelial cells but also that the addition of fibroblasts has a modulating effect. What exactly causes these differences in IL-8 response is impossible to say at this moment and needs further investigation.

6.5.4 Challenges of Calu-3 submerged monocultures and mono- and co-cultures at ALI with LPS

Calu-3 mono-cultures under submerged conditions responded to all three types of LPS with increased IL-8 secretion. *B. cepacia* LPS stimulated IL-8 secretion at a concentration as little as 1 ng/ml, whereas the other two LPSs from *P. aeruginosa* needed to be at a concentration of 100 – 1000 ng/ml, which fits with reports stating that *B. cepacia* LPS of J2315 is more potent than LPS of *P. aeruginosa* (Zughaier *et al*., 1999). In contrast, when these cells were cultured at ALI and were stimulated with 1000ng/ml LPS the responses were different as we only see an induction of IL-8 secretion after exposure to *P. aeruginosa* 50 DR LPS, with the CF isolate being more potent. In this case significant increase of IL-8 was seen after all three challenges in the apical supernatant collected. Comparing results of ALI mono-culture and ALI co-culture, again the results are completely different as no induction of IL-8 was observed at all in co-cultures of HPF-

Calu-3. HPF mono-cultures on TWs did respond to *P. aeruginosa* 10 LPS, when these were challenged from both sides. They might not be as responsive in the co-culture challenges as they only encounter LPS from the basolateral side and have Calu-3 as a protective tight epithelial layer on the top. However, HPF mono-cultures did not show a response to *B. cepacia* and *P. aeruginosa* 50DR LPS, which suggests that HPF do not respond here either. Why Calu-3 in co-culture do not respond to *P. aeruginosa* 50DR LPS is not exactly known yet. PRR expression has not been analysed for HPF and Calu-3 after stimulation or on TWs and makes it impossible to comment on possible TLR-4 translocation to the membrane surface and whether on Calu-3 there is a difference in apically or basolaterally receptor expression.

For submerged unstimulated Calu-3 it has been shown that CD14 is surface expressed but not TLR-4. The expression of MD-2 was not observed here but another group has analysed mRNA expression of these three receptors and found that all of them are expressed in Calu-3 cultured at ALI (O'Grady, 2007). These were mRNA analyses and did not define membrane and intracellular expression but based on these finding and results presented here it is assumed that Calu-3 are able to respond to LPS via this signalling pathway. The huge difference in response of submerged mono-cultures to *B. cepacia* compared to mono-cultures at ALI, which do not respond to this type is interesting as submerged cultures respond to very low levels of *B. cepacia* LPS. This indicates that responses of epithelial cells that are well differentiated are very different and submerged cultures might not reflect at all, what affect LPS and other stimuli have on epithelial cells *in vivo*.

For Calu-3 mono-cultures and HPF-Calu-3 co-culture the responses are quite different and identification of receptor expression of both cell types could shed some more light on these differences. It is clear though that HPF do modulate the response of Calu-3 to LPS and further investigation should elucidate the meaning and importance of cell-to-cell communication.

All together HPF mono-cultures were observed to respond to different types of LPS under submerged conditions and when grown on TWs. How HPF physiology is affected by the different culture conditions needs to be further analysed but it has been shown that they did form a multi-cell layer rather than a mono-layer, when grown on TWs, which could explain the differences of IL-8 concentrations observed.

Presumably this was the same for epithelial cell lines used. Surface expression of CD14 and TLR-4 has only been analysed for cells grown under submerged conditions and in an unstimulated manner. However, there is a significant difference in responses to LPS comparing these cell

models, and therefore these need further investigation in terms of receptor expression, for example. Especially further investigation of the co-culture models is needed to analyse how the addition of HPF modulate the epithelial cell response to LPS as these were different comparing mono- and co-culture models. This was also shown for Calu-3 in this chapter. The responses of mono- compared to co-cultures did have completely different outcomes in terms of IL-8 secretion and emphasizes that cytokines and their receptors are crucial for these cellular responses and functions. However, variations found among these epithelial cell lines, especially comparing C38 and IB3-1 to Calu-3, in terms of inflammatory response by secreting IL-8 could be due to different genotypic background of these epithelial cells, which originated from different diseases. Other factors that might play a role are differences in CFTR expression levels for example and results might be cell type specific.

Co-culture models are needed to elucidate the cell-to-cell communication and identify the constantly ongoing interactions and how these are related to cystic fibrosis. This might lead a way to new strategies for manipulating clinical settings.

7 Chapter 7 Inflammatory response to intact bacteria

7.1 Introduction

Morbidity and mortality of CF patients is largely due to lung disease, which is caused to a large extent by the chronic, lifelong bacterial infection and concomitant neutrophilic inflammation. Some of the hallmarks of the CF lung, such as impaired mucociliary clearance and accumulation of highly viscous mucus, participate in the increased susceptibility to bacterial colonisation and provide an environment that can be readily infected. In early stages of CF *Staphylococcus aureus* (*S. aureus*) is one of the most common pathogens to be isolated and is the most prominent bacterium in children and in early adolescents. Later on, *P. aeruginosa* is found the most prominent pathogen in the CF lung (by the age of 18 about 80 % of patients are chronically infected) and most mortality is accounted to this well established bacterium (Burns *et al*., 1998).Next to these two bacteria there are others found specifically in the CF lung, such as *B. cepacia,* which is known to be transmissible between patients and usually leads to a rapid decline in lung function (Govan *et al*., 1993). All three of these bacteria are regularly found in CF patients, with *P. aeruginosa* being the most common one in adulthood (de Bentzmann *et al*., 1996c), *B. cepacia* being a very destructive one (Drevinek and Mahenthiralingam, 2010) and *S. aureus*, which is usually found in infants and young children but is also found in 40% of CF adult patients next to *P. aeruginosa* (Kahl, 2010, Burns *et al*., 1998).

Bacterial infections have been studied extensively *in vivo* and *in vitro* but the tremendous amount of variables make it difficult to link single factors to the pathogenesis of CF, which is more likely to be multifactorial. There are several organisms found in CF lungs and a lot of them have been isolated and found to be different strains with different genotypes and phenotypes (Razvi *et al*., 2009). These can differ in their expression of virulence factors and also it has been shown that some bacteria significantly change to adapt to the harsh CF lung environment to maintain infection (Smith *et al*., 2006). *In vitro*, the main attention has been directed towards analysing the response of the epithelial cells of the airways (Zhang *et al*., 2005, Kube *et al*., 2001, da Silva *et al*., 2004, Martin and Mohr, 2000) and there are few co-culture models that investigate cell-to-cell communication and the multi-cellualr response to relevant pathogens. Additionally there is very little research published on the role of pulmonary fibroblasts in the extensive pro-inflammatory state characteristic of the CF airways. There is a need for multicellular models and throughout this work a multicellular non-CF as well as a multicellular CF model are presented, which will help to investigate multi cell responses to CF relevant pathogens to fill gaps in the puzzle of understanding CF pathogenesis.

Bacterial infections are initially recognised by the host through pathogen associated molecular patterns (PAMPs), which are presented to the host epithelial and immune cells, and which usually initiate a pro-inflammatory response. These PAMPs can be of different nature, while LPS is found in the membrane of gram-negative bacteria, such as *P. aeruginosa* for example (Raoust *et al*., 2009), peptidoglycan (PGN) and Lipoteichoic acid (LTA) are PAMPs in the bacterial cell walls of gram-positive bacteria, such as *S. aureus* (Fournier and Philpott, 2005). The ensuing inflammatory response involves the production and release of pleitropic cytokines such as IL-6 and TNF-alpha. In addition, for CF airways disease pathogenesis, the most important chemokine secreted is IL-8. IL-8 is a potent neutrophil chemoattractant; sustained release of this chemokine causes an excessive influx of activated neutrophils, which in turn take their part in the cycle of inflammation by releasing more inflammatory mediators (e.g. elastase).

Pattern recognition receptors (PRR), which are located on the host's cell surface membranes, recognise PAMPs. Typical PRRs are the toll-like receptors, such as TLR-4 and TLR-2. TLR-4 recognises bacterial LPS of gram-negative bacteria, whereas TLR-2 recognises lipoproteins and PGN of gram-positive bacteria (Hauber *et al*., 2005), for example. In both cases these TLRs lead to NF-κB activation and its translocation to the nucleus, where target genes are then transcribed, such as the gene for IL-8.

In this chapter *S. aureus*, *B. cepacia* and P aeruginosa were used, as live organisms, to induce a pro-inflammatory response in the characterised mono- and co-culture systems in order to and investigate cellular responses to these bacteria in their intact and infectious state.

Host cell viability was measured after a 24 h exposure of the mono-and co-culture systems to these live bacteria, as well as the secretion of the key chemokine in CF lungs, IL-8. Furthermore TER measurements were used as an indicator of epithelial integrity; this is one of the main functions of the airways in innate immunity.

7.2 Methods

7.2.1 Cell viability assay using Cell Titer Blue™

Cell Titer Blue™ (CTB) is an endpoint assay that provides a homogeneous, fluorometric method to monitor cell viability. Viable cells, which are metabolically active, can convert resazurin (blue with little intrinsic fluorescence activity) into its highly fluorescent product, resorufin (pink; detectable with 560 nm excitation and 590 nm emission). CTB was directly added to the cell medium at dilution (1:5) after live bacteria have been exposed to gentamicin for 1 h and the plate was then incubated for 2 h at 37 °C prior to analysis. The fluorescent intensity was measured on a standard multiwell fluorescent plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with a 560 nm excitation, and 590 nm emission wavelength. CTB was used in different experimental layouts, which will be discussed individually in the chapters affected.

7.2.2 Transepithelial electrical resistance (TER) measurements

TER of mono- and co-cultures on TWs was measured using an Epithelial Voltohmeter with STX2 chopstick electrodes (World Precision Instruments) to monitor confluence and barrier formation of the epithelial cell layer. TER was measured on day 14 or just before exposure to live bacteria to verify epithelial cell confluence and intactness of epithelial cell layer and TJ formation.

TER values reported in this work take into account the area of the cell layer (area of TWs = 0.33 cm^2) and are expressed as $\Omega \times cm^2$. Triplicate measurements were taken for each TW and the background resistance, which was typically between 100-120 $\Omega \times cm^2$ (cell-free collagen IV coated TW) was subtracted from the average of a triplicate measurement.

TER was measured immediately before and straight after the 24 h incubation with live bacteria to analyse whether cell layer integrity was affected compared to the control TWs.

7.2.3 Bacterial culture

P. aeruginosa 50DR*, B. cenocepacia* J2315 and *S. aureus* (ATCC 6538) were initially grown on MHA plates in a 37°C incubator. Colonies of *P. aeruginosa* 50DR*, B. cenocepacia* J2315 and *S. aureus* were then picked from a freshly cultivated MHA plate and grown in 50 ml MHB at 37 °C overnight in a shaker at 150 rpm. On the next day cultures were spun down at 3500 x g for 15 min and washed in 10 ml PBS. This wash was repeated three times before the optical density (OD) was measured at 470 nm. The OD was then adjusted to 1, which corresponds to approximately 1 x 10^9 CFU/ml for *P. aeruginosa, S. aureus* and *B. cepacia.* These bacterial

suspensions were then diluted 1:10 to make a stock solution of 1 x 10^8 CFU/ml. The final bacterial cell density used in the experiments under submerged conditions or at ALI was 1 x 10^7 CFU/ml.

7.2.4 Antibiotic susceptibility of *S. aureus*, *B. cepacia* and *P. aeruginosa*

In order to prove that the applied cell viability test (CTB) and the conversion of CTB from non-fluorescent to fluorescent was down to the epithelial cells it was necessary to determine the susceptibility of *P. aeruginosa* 50DR*, B. cenocepacia* J2315 and *S. aureus* (ATCC 6538) to the antibiotic gentamicin to validate the killing affect of this antibioticum. Small filter paper discs containing 200µg/ml gentamicin were placed onto MHA plates, which were covered with bacterial suspension using a swab to generate a bacterial lawn. If the bacteria applied were sensitive to gentamicin, a clear zone of growth inhibition was seen around the gentamicin containing filter. Control plates were set up and filters with PBS were added to the plate. No clear zone was observed here (data not shown). A similar experiment was carried out with Normocin, an antibiotic routinely used in cell culture media. In this case agar plates were prepared all at the same volume and holes were punched into the agar, which were then filled with 50µl of normocin (either 50 mg/ml stock solution or at working concentration 100µg/ml diluted in full growth media, one for each type) after the bacterial suspension was spread using a swab. Bacteria need to be spread before because otherwise the corners of the punched holes possibly catch the swab and a strong inoculate will colonize there. Again PBS was used a negative control.

7.2.5 Treatment of submerged mono-cultures and mono- and co-cultures grown at ALI with live bacteria

Submerged mono-cultures were set up as described in 2.17. Cells were grown in SF medium, which contains ITS, for 24 h before bacteria were added. ITS-medium containing approximately 1 x 10^7 CFU/ml of live bacteria were then added and cells were incubated for 24 h. Apical supernatant and basolateral medium samples were then collected and stored at -80°C for IL8 ELISA analysis. Cell cultures were incubated with ITS-medium containing 100 µg/ml gentamicin for one hour. After the gentamicin treatment, the medium was removed and ITS-medium containing CTB was added to assess cell viability.

The same procedure was followed to expose mono-and co-cultures grown at ALI. After 14 days of culture three TWs per experiment were challenged either apically, basolaterally or simultaneously from both sides with live bacteria at 1 x 10^7 CFU/ml. Samples were collected from each TW from the apical side and from the basolateral side and were independently analysed for IL-8 release.

7.2.6 Detection of Interleukin 8 (IL-8) by ELISA

For the quantification of human IL-8 in cell culture supernatants of submerged and ALI mono- and co-cultures treated with live bacteria, a human IL-8 ELISA development kit, was purchased from Peprotech EC Ltd. (London, UK). All reagents were part of the kit unless otherwise stated and were prepared according to manufacturer's product information. IL-8 ELISA protocol is described in detail in 2.19.

7.2.7 Statistical analysis

Throughout this chapter statistical analysis of results and significant differences were determined by 1Way-Anova followed byTukey's multiple comparison test. Results are presented as mean ± SD. Results were considered significant when $p \leq 0.05$ = *; $p \leq 0.01$ = **; $p \leq 0.001$ = ***.

7.3 Aims

In this chapter submerged mono-cultures as well as mono-and co-cultures at ALI were exposed to live *S. aureus*, *B. cepacia* and *P. aeruginosa* in order to investigate the response of the *in vitro* models to these common, life-threatening CF pathogens and to compare results with other published literature to determine functionality of these models and how well they reflect the CF airways.

In order to achieve these aims it was necessary to analyse host cell viability after exposure to live bacteria.

Additionally the cell layer integrity was monitored by measuring TER before and after the challenges

Measurement of the concentration of CF's key chemokine IL-8 after exposure to live bacteria

7.4 Results

7.4.1 Cell viability and IL-8 release of submerged mono-cultures after exposure to live bacteria

7.4.1.1 Exposure of submerged HPF to live bacteria

HPF in submerged mono-cultures were exposed to live bacteria for 24 hours. The bacteria used were *S. aureus, B. cepacia* and *P. aeruginosa* and out of these only *P. aeruginosa* had an effect on HPF cell viability (figure 7.1 A). The baseline fluorescence of HPF when exposed to medium only was 1597.6 ± 28.34 FU and after a 24 h exposure to live *P. aeruginosa* it was reduced to 807.74 ± 317.42 FU, a 1.9 fold decrease.

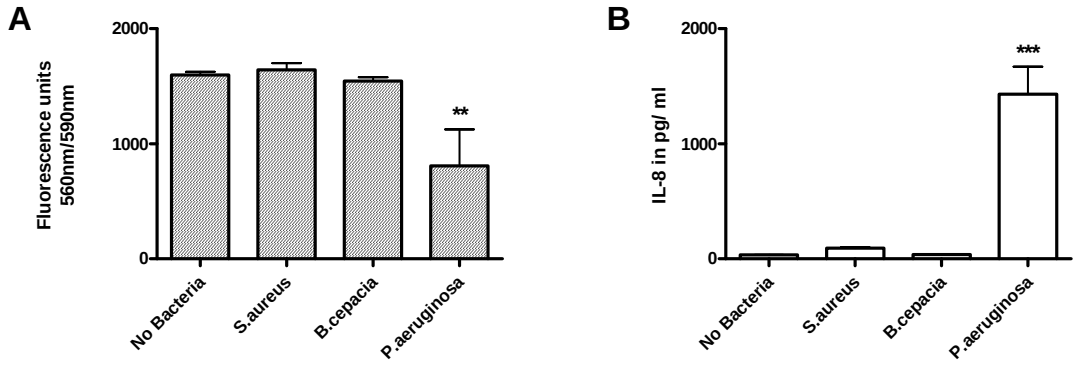

Figure 7.1 Cell viability and IL-8 release of HPF after exposure to live bacteria
HPF were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. Afterwards HPF were exposed to 1 x 10^7 cfu/ml for 24 h. Supernatants were collected and analysed for IL-8 concentrations and HPF were assessed for cell viability after bacteria were exterminated using 100µg/ml gentamicin for 1 h. On HPF, only *P. aeruginosa* exposure caused a significant decrease of cell viability and also a tremendous increase in IL-8 concentration. Data are presented as mean ± SD from 3 individual experiments (n=4).

It is interesting to note that, even though there was a *P. aeruginosa*-dependent decrease in cell viability there was an significant increase in IL-8 secretion after 24 h exposure to this bacterium (figure 7.1 B). IL-8 concentration of control supernatants was 35.95 ± 1.5 pg/ml compared to 1431.44 ± 239.77 pg/ml in cells exposed to *P. aeruginosa.* This is almost a 6-fold increase, even without taking into account the decrease in viability. No significant changes in IL-8 release were observed in response to *S. aureus* or *B. cepacia*.

7.4.1.2 Exposure of submerged C38 to live bacteria

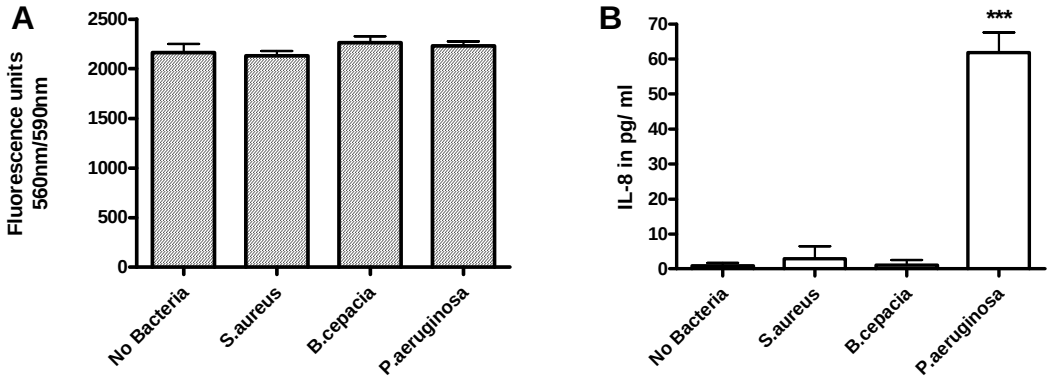

Figure 7.2 Cell viability (A) and IL-8 release (B) of C38 submerged mono-cultures after exposure to live bacteria
C38 were seeded onto a 24-well plate at 1 x 10^5 cells per well and incubated for 24 h before they were serum starved for 24 h. Afterwards C38 were exposed to 1 x 10^7 cfu/ml bacterial suspensions for 24 h. Supernatants were collected and analysed for IL-8 concentrations and the cells were assessed for cell viability after bacteria were killed using 100µg/ml gentamicin for 1 h. For C38, none of the live bacteria used had an effect on cell viability but exposure to *P. aeruginosa* caused a significant increase in IL-8 secretion into the supernatant. Data are presented as mean ± SD from 3 individual experiments (n=4 replicates).

Cell viability (figure 7.2 A) of C38 was not affected by exposure to live *S. aureus*, *B. cepacia* or *P. aeruginosa*. No significant changes of FU could be observed compared to the control, C38 without bacteria which had a baseline fluorescence of 1597.60 ± 28.35 FU. When IL-8 secretion (figure 7.2 B) was analysed, a significant increase in comparison to control was noted after a 24 h exposure of C38 to live *P. aeruginosa*. The IL-8 secretion in the control wells was at an average of 0.96 ± 0.77 pg/ml, which increased to 61.85 ± 5.78 pg/ml after exposure to live *P. aeruginosa*.

7.4.1.3 Exposure of submerged IB3-1 to live bacteria

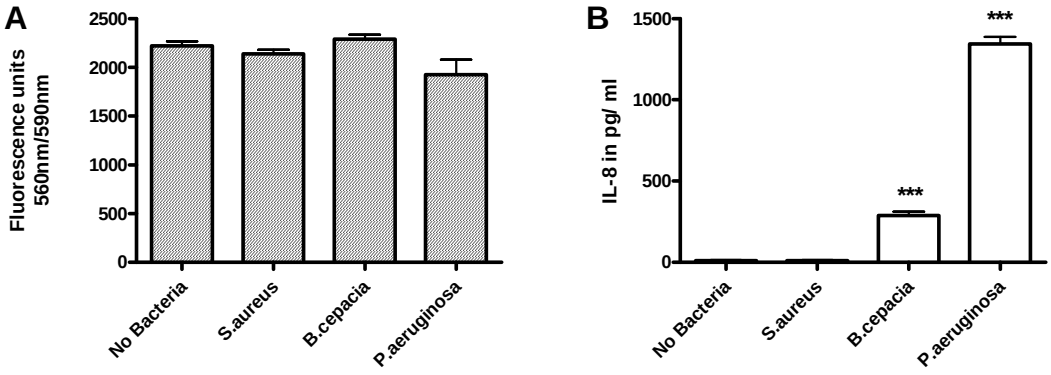

Figure 7.3 Cell viability (A) and IL-8 release (B) of IB3-1 after exposure to live bacteria
IB3-1 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. IB3-1 were then exposed to 1 x 10^7 cfu/ml of each bacterium for 24 h. Supernatants were collected and analysed for IL-8 concentrations. Cell viability was assessed after bacteria were killed using 100µg/ml gentamicin for 1 h. IB3-1 did not show any significant decrease of cell viability after a 24 h incubation with these live bacteria. IL-8 secretion by IB3-1 was significantly increased following exposure to either *B. cepacia* or *P. aeruginosa*.
Data are presented as mean ± SD from 3 individual experiments (n=4 replicates).

The exposure of IB3-1 submerged mono-cultures to *S. aureus, B. cepacia* and *P. aeruginosa* did not show any effect on cell viability (figure 7.3 A). The control mean FU measured in the viability assay was 2219.58 ± 46.53 FU. There was a slight decrease of cell viability after the exposure to *P. aeruginosa,* with average FU of 1924.88 ± 152.33 but this was not significantly different to the control. IL-8 secretion by IB3 cells (figure 7.3 B) was significantly increased by exposure to *B. cepacia* and *P. aeruginosa*. IL-8 secretion of unstimulated IB3-1 cells was 9.93 ± 1.98 pg/ml compared to 286.63 ± 25.13 pg/ml after exposure to *B. cepacia* and to 1342.54 ± 45.74 pg/ml after exposure to *P. aeruginosa*.

7.4.1.4 Exposure of submerged Calu-3 to live bacteria

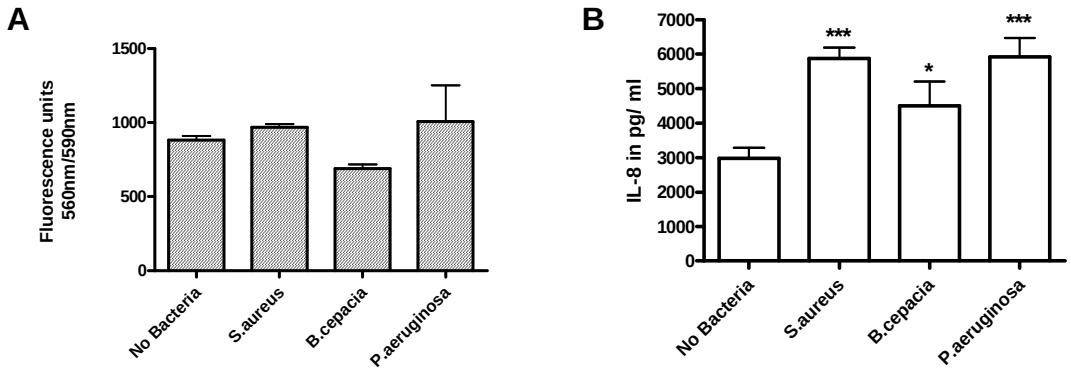

Figure 7.4 Cell viability (A) and Il-8 release (B) of Calu-3 after exposure to live bacteria
Submerged mono-cultures of Calu-3 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved and then exposed to live bacteria (1 x 10^7 cfu/ml) for 24 h. Supernatants were collected and analysed for IL-8 concentrations. Cell viability was assessed after bacteria were killed using 100µg/ml gentamicin for 1 h. Calu-3 did not show any significant decrease of cell viability after 24 h incubation with these live bacteria. IL-8 secretion by Calu-3 was significantly increased following incubation with *S. aureus B. cepacia* or *P. aeruginosa*. Data are presented as mean ± SD from 3 individual experiments (n=4 replicates).

After 24 h exposure to live *S. aureus, B. cepacia* or *P. aeruginosa,* no significant changes in cell viability (figure 7.4 A) could be observed for Calu-3 when grown under submerged conditions. This is different when IL-8 concentrations (figure 7.4 B) were analysed. Significant increases were seen after exposure to all three different bacteria. The control supernatant of Calu-3 without exposure to bacteria contained 2975.33 ± 304.6 pg/ml of IL-8 compared to 5869.97 ± 314.58 pg/ml of IL-8 after exposure to live *S. aureus* for 24 h. IL-8 concentration observed after exposure to *B. cepacia* was 4501.53 ± 696.12 pg/ml and was 5923.42 ± 549.9 pg/ml following challenge with *P. aeruginosa*.

7.4.2 Cell viability and inflammatory response of mono- and co-cultures at ALI after exposure to live bacteria

7.4.2.1 Exposure of submerged HPF mono-cultures grown on TWs to live bacteria

HPF were grown on TWs under submerged conditions for 14 days before these were challenged with live *S. aureus* from either the apical side, the basolateral side or simultaneously from both sides and were then assessed for cell viability (fig 7.5 A), TER (B) of the mono-culture and IL-8 secretions (C) after the 24 h challenge.

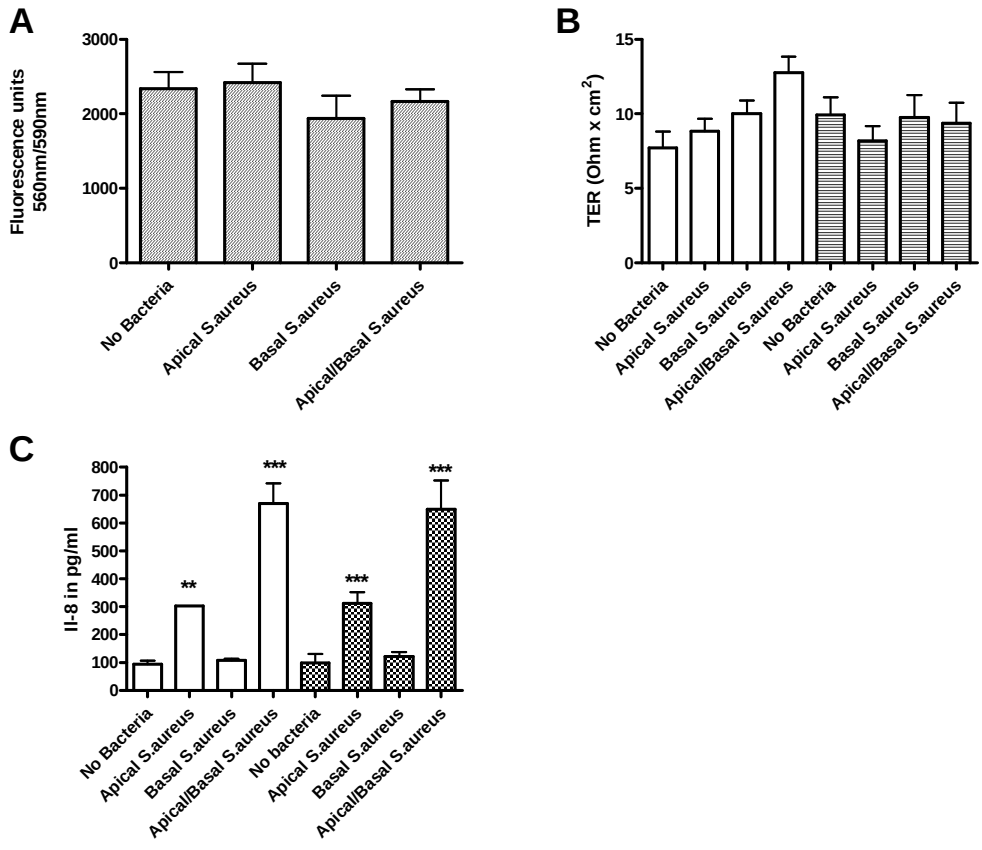

Figure 7.5 Cell viability (A), cell layer integrity (B) and inflammatory response (C) of HPF after exposure to *S .aureus*

HPF were grown under submerged conditions on TWs for 14 days before these were challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides simultanously, excluding the control TWs. Cell viability was not effected significantly, nor was TER measured before (clear bars) and directly after (striped bars) the 24 h incubation with live *S. aureus*. Significant increases of IL-8 concentrations were observed for apically challenged as well as simultaneously (apical and basal) challenged HPF. This increase was observed in both apical (clear bars) and basolateral (checked graphs) samples analysed. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

In figure 7.5 cell viability (A) is shown for HPF after exposure to *S. aureus* and no significant differences were seen for any of the three challenges when compared to the control, which showed a baseline fluorescence of 2304.27 ± 143.00 FU. Graph B represents the results for TER (B) measured before (clear bars) and after (striped bars) the challenges. For HPF, which were shown before not to build up a high electrical resistance, the average TER of the control TWs before the challenge was 7.74 ± 1.06 Ω x cm^2 and was 9.94 ± 1.166 Ω x cm^2after the challenges. IL-8 secretion (C: apical samples =clear bars, basolateral samples =checked bars), however, was significantly increased following apical challenge with live *S. aureus* as well as for simultaneous challenge from both sides. HPF on their own without exposure to live *S. aureus* secreted 94.54 ± 12.68 pg/ml to the apical side and 99.16 ± 31.82 pg/ml in the basolateral medium, which increased to 302.15 pg/ml in the apical supernatant and to 312.59 ± 39.74 pg/ml in the basolateral compartment after the apical challenge with live *S. aureus*. There is almost equal concentrations of IL-8 found on both sides of the cell layer, which is also true for the increased IL-8 secretion after the simultaneous challenge from both sides. In this case the apical supernatant contained 671.14 ± 70.52 pg/ml of IL-8 and the basolateral medium contained 649.95 ± 102.06 pg/ml.

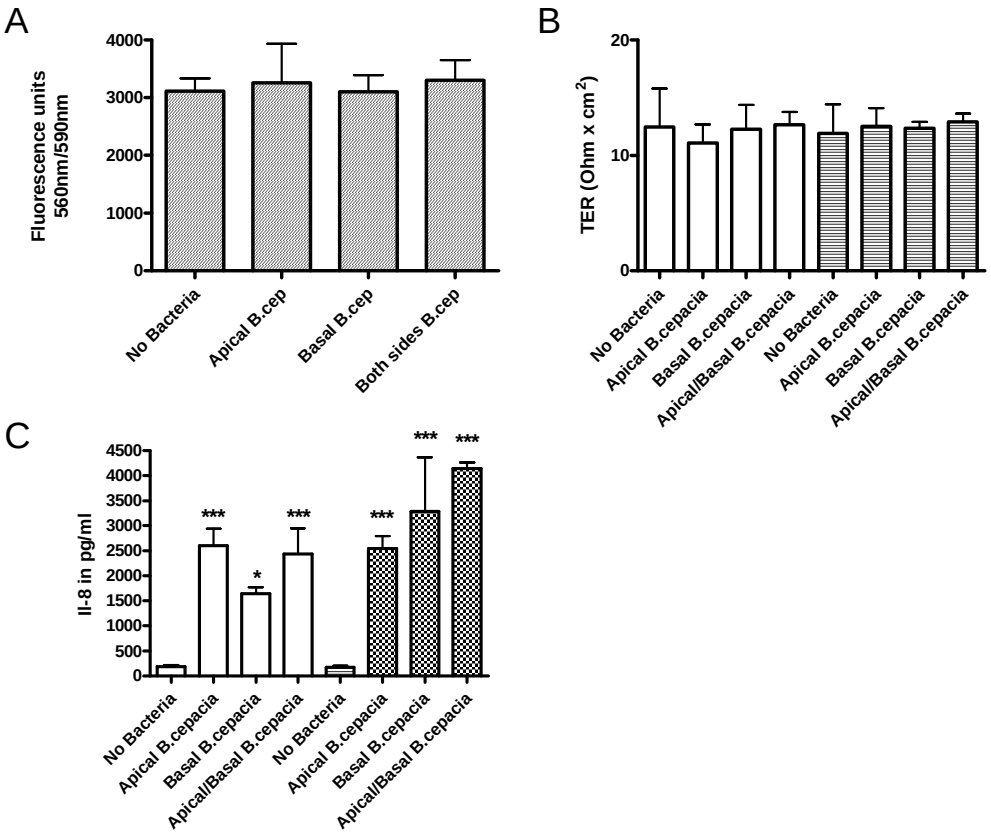

Figure 7.6 Cell viability (A), TER (B) and IL-8 release (C) of HPF after exposure to live *B. cepacia*
HPF were grown under submerged conditions on TWs for 14 days before challenge with live *B. cepacia*, which was applied either apically, basolaterally or from both sides simultaneously,. Cell viability of HPF mono-culture was not affected by the presence of live *B. cepacia* and nor was the TER, which was measured before (clear bars) and directly after (striped bars) the 24 h incubation with live *B. cepacia*. IL-8 concentrations, however, were significantly increased in all apical supernatants (clear bars) and basolateral medium (checked bars), irrespective of the site of the challenge. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF mono-cultures were grown under submerged conditions on TWs for 14 days before they were challenged with live *B. cepacia*. The control TWs were treated the same as all others but without bacteria, whereas the TWs that were challenged were exposed to live *B. cepacia* from either the apical side, the basal side or they were exposed to bacteria on both sides at the same time. HPF's cell viability (figure 7.6 A) was not changed after a 24 h incubation with live *B. cepacia* (baseline fluorescence 3107.46 ± 225.35 FU) and this was also found for the TER (B) measured before (clear bars) and after (striped bars) the challenge. The control TWs showed an average TER of 12.47 ± 3.33 Ω x cm^2 before the challenge and a TER of 11.92 ± 2.51 Ω x cm^2.IL-8 secretion (C), however, was significantly increased after each challenge and this was true for apical supernatants (clear bars) as well as basolateral medium (checked bars). The control TWs

had an average of 190.21 ± 29.62 pg/ml in the apical compartment and 166.42 ± 40.9 pg/ml in the basal medium. For the apical challenge 2599.38 ± 343.84 pg/ml IL-8 was found apically and 2543.67 ± 244.25 pg/ml was found basally. When HPF were challenged on the basolateral side only, the apical supernatant contained 1637.69 ± 135.89 pg/ml and the basolateral medium contained 3286.86 ± 1081.48 pg/ml IL-8 and when cells were challenged on both sides there was 2431.85 ± 515.65 pg/ml IL-8 in the apical supernatant and 4143.27 ± 117.57 pg/ml IL-8 in the basal medium.

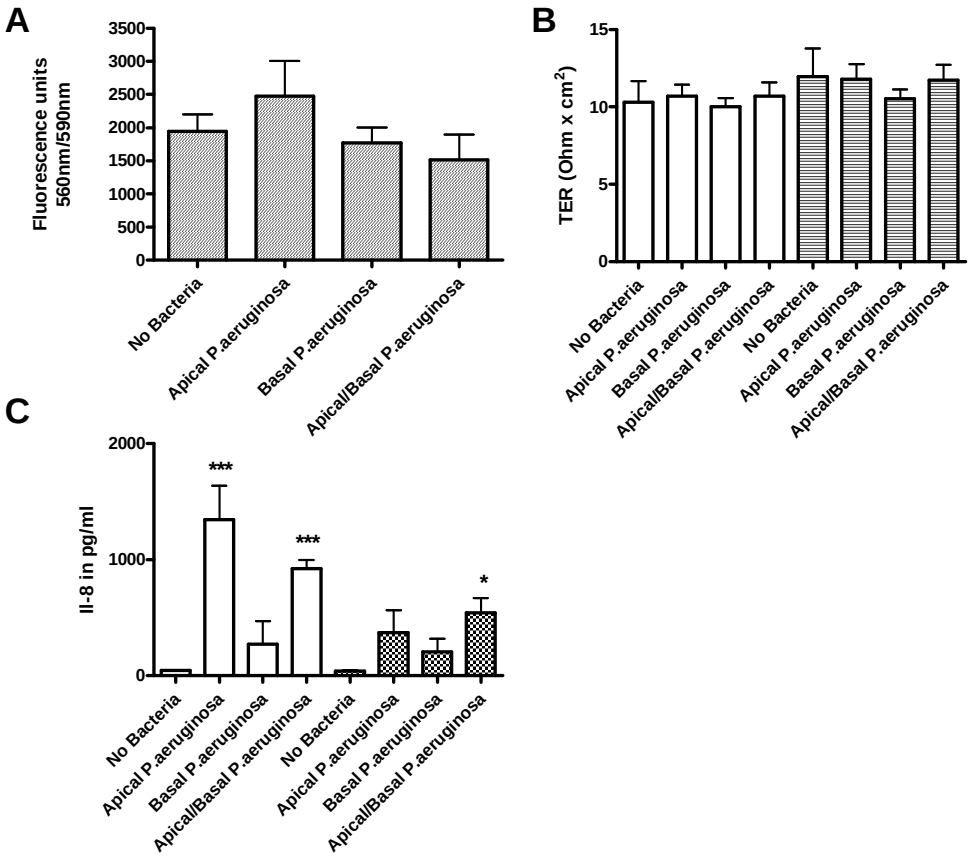

Figure 7.7 Cell viability (A), TER (B) and IL-8 release (C) of HPF mono-culture after exposure to live *P. aeruginosa*

HPF mono-cultures were grown submerged on TWs for 14 days before these cells were exposed to live *P. aeruginosa*, which were applied either apically, basally or simultaneously from both sides. Cell viability after 24 h exposure to live bacteria was not significantly affected compared to the control TWs, which were handled the same but without the exposure to bacteria. TER was measured before (clear bars) and after (striped bars) the experiment but no significant difference was observed after 24 h incubation with live *P. aeruginosa*. IL-8 release of HPF mono-culture, however, was significantly increased by live bacteria applied apically and also when applied simultaneously on both sides. This was seen in the apical supernatants (clear bars), whereas in the basal medium samples (checked bars) the increase noted was only significant when challenged from both sides. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

Mono-cultures of HPF were exposed to live *P. aeruginosa* after 14 days culture on TWs. Cell viability (figure 7.7 A) was analysed after a 24 h incubation with live bacteria and was found to not be affected significantly by this event. HPF control mono-cultures showed a baseline fluorescence of 1943.62 ± 254.59 FU. These cultures were also monitored in terms of changes in electrical resistance and the TER (B) was measured before (clear bars) and after (striped bars) the 24 h incubation. For the control TWs the TER was 10.30 ± 1.38 Ω x cm^2 before the challenge and it was 11.95 ± 1.82 Ω x cm^2. No significant changes were observed for apically, basally or simultaneous challenged HPF with live *P. aeruginosa*. Furthermore the IL-8 concentration (C) in apical supernatants (clear bars) and basal medium samples (checked bars) was analysed. The exposure to live *P. aeruginosa* caused a significant increase of IL-8 in apical supernatants, when HPF were challenged apically or apically and basolaterally at the same time. The control TWs showed IL-8 concentrations of 45.38 ± 1.49 pg/ml in the apical supernatant and 39.28 ± 9.04 pg/ml in the basal medium compared to 1344.54 ± 291.54 pg/ml in apical supernatant and 371.5 ± 192.48 pg/ml in the basal medium, which was increased but not significant. Both samples of the simultaneous challenge were significant and increased to 922.38 ± 124.18 pg/ml on the apical side and to 540.2 ± 126.84 pg/ml in the basal compartment.

7.4.2.2 Exposure of C38 mono-cultures to live bacteria

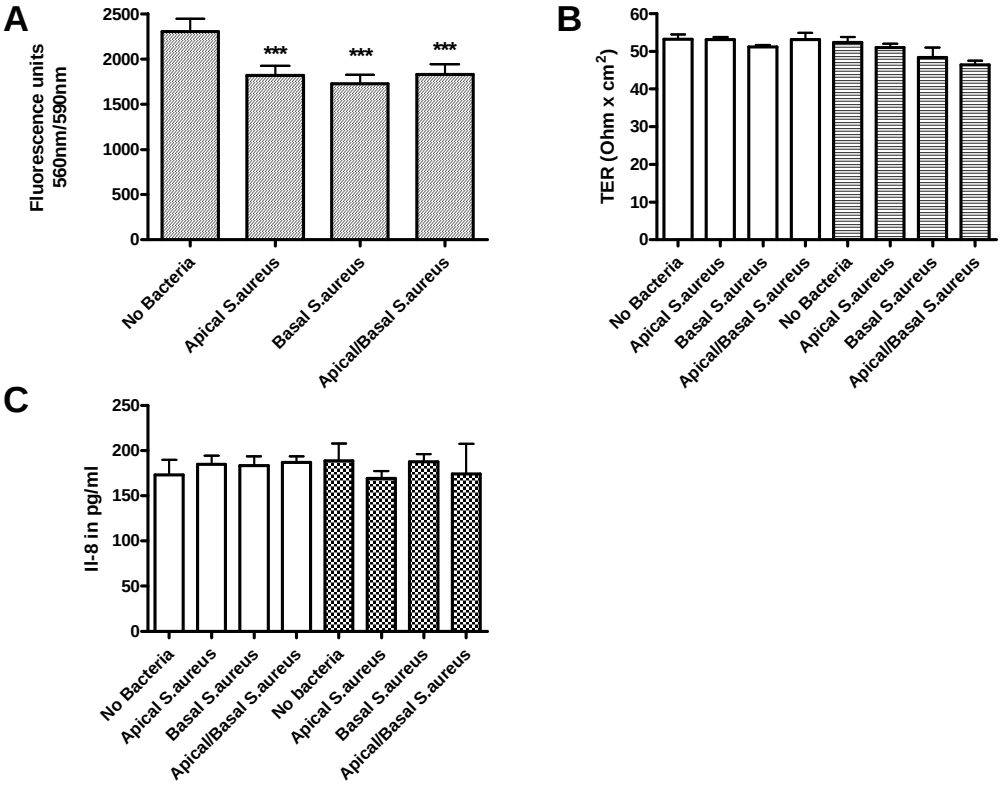

Figure 7.8 Cell viability (A), TER (B) and IL-8 release (C) of C38 grown at ALI after exposure to *S. aureus*
C38 were grown on TWs at ALI for 14 days before they were challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were C38 without bacteria but medium was applied apically to create similar conditions. Cell viability was significantly decreased in all three challenges when compared to the control. The measured TER after the challenges (striped bars) was not significantly different compared to before (clear bars) and nor was the IL-8 concentration. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38 mono-cultures grown at ALI were analysed after they were exposed to live *S. aureus*. Live bacteria decreased cell viability (figure 7.8 A) significantly when applied apically, basolaterally or from both sides at the same time. The average FU measured for control TWs was 2304.26 ± 143.00 FU compared to 1817.44 ± 107.27 FU after apically treatment with live *S. aureus.* When *S. aureus* was applied basolaterally cell viability was reduced to 1726.23 ± 101.7 FU and similarly lowered to 1830.2 ± 109.7 FU after simultaneous challenge on both sides. Interestingly the cellular integrity was not directly affected and no significant differences were identified when comparing TER (B) to control TWs before (clear bars) and after (striped bars) the challenges. The control mono-cultures had an TER of 53.27 ± 1.23 Ω x cm^2 before the 24 h incubation and they

had a TER of 52.43 ± 1.38 Ω x cm^2 after this challenge. IL-8 release into either apical (clear bars) supernatants or the basolateral compartments (checked bars) did not change for any of the challenges. The baseline IL-8 secretion (figure 7.8 C) was 173.03 ± 17.0 pg/ml in the apical sample (clear bars) of the control and 188.37 ± 19.65 pg/ml in the basaolateral medium (checked bars).

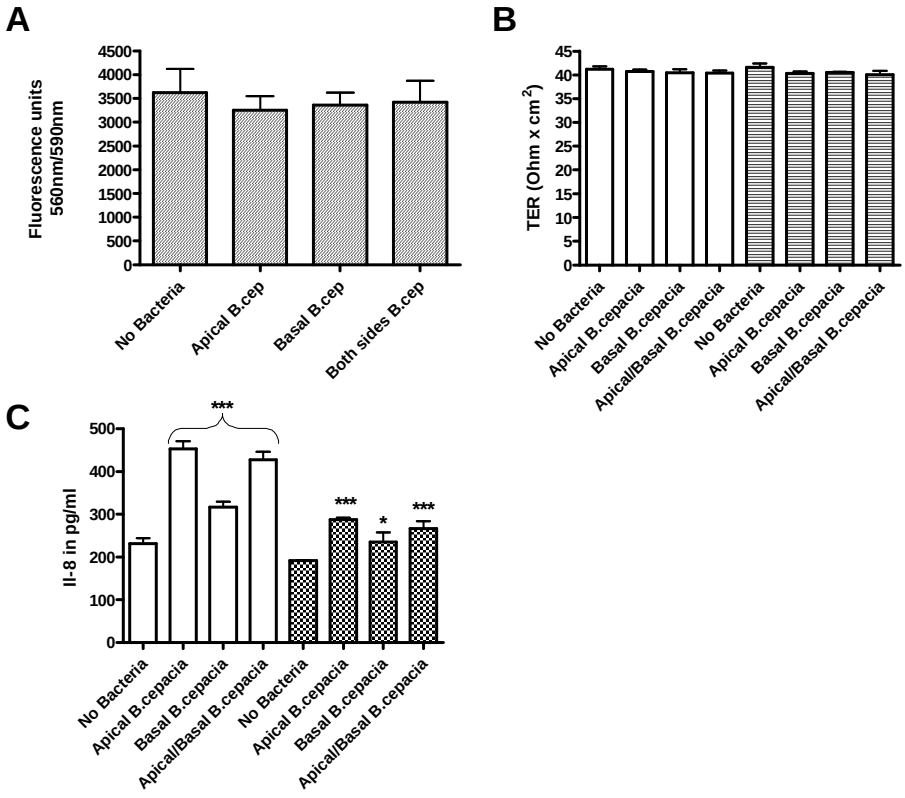

Figure 7.9 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to live *B. cepacia*
C38 grown at ALI were exposed to live *B. cepacia*, which was applied either apically, basolaterally or from both sides simultaneously. Untreated TWs, which were only challenged with growth medium and no bacteria served as control. Cell viability (A) and cell layer integrity (B) were not affected by the presence of live *B. cepacia*. IL-8 concentrations, however, were significantly increased after all three different exposures and in apical supernatants (clear bars) as well as in the corresponding basolateral media collected (checked bars) compared to the control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

C38 mono-cultures did not show any change in cell viability (figure 7.9 A) after exposure to live *B. cepacia* for 24 h, which was either applied apically, basolaterally or on both sides simultaneously. The baseline fluorescence measured for the control was 3619.74 ± 502.29 FU.

The exposure to live *B. cepacia* did not have a direct influence on TER (figure 7.9B), when comparing the TERs from before (clear bars) and the TER after the challenges (striped bars). TER of control TWs was 41.14 ± 0.69 Ω x cm^2 before the challenge and was 41.54 ± 0.85 Ω x cm^2. The third aspect that was analysed was IL-8 concentration (figure 7.9C), which was investigated in apical supernatants (clear bars) as well as in the basal media collected (checked bars). IL-8 release was increased significantly in apical as well as in basal samples. The control TWs had an IL-8 concentration of 231.33 ± 12.42 pg/ml in the apical supernatant and 191.26 ± 1.35 in the basal compartment. After the apical challenge 452.43 ± 18.33 pg/ml IL-8 was found in the apical supernatant and 287.56 ± 3.9 pg/ml IL-8 was found in the basal medium. For the basal challenge these were 316.53 ± 13.45 pg/ml IL-8 apically and 234.6 ± 22.49 pg/ml IL-8 basolaterally. When challenged from both sides values were similar to the apical challenge at 426.85 ± 19.12 and 267.02 ± 17.40 pg/ml IL-8 for the apical and basal supernatants, respectively.

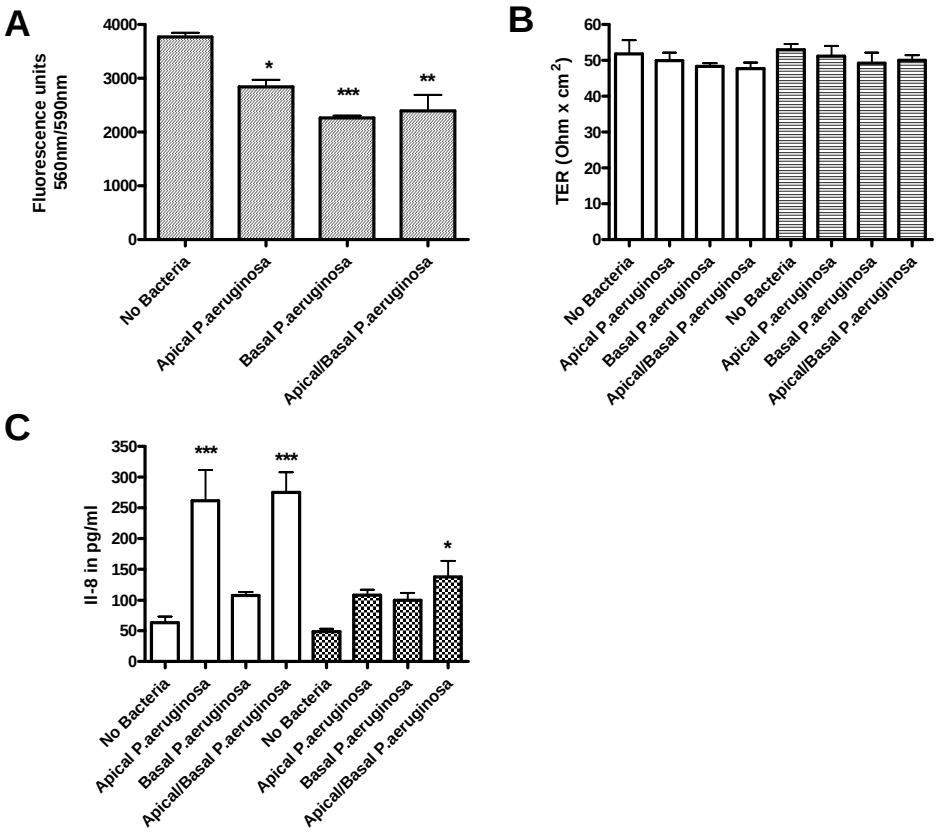

Figure 7.10 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to live *P. aeruginosa*

C38 mono-cultures were grown at ALI for 14 days and were then exposed to live *P. aeruginosa*, which was applied either apically, basolaterally or from both sides simultaneously. Untreated TWs, which were treated the same but without bacteria served as control. Cell viability (A) of C38 was significantly decreased by all three different challenges, whereas the TER (B) was not affected by this exposure to live *P. aeruginosa*. IL-8 concentrations, however, were significantly increased in the apical supernatant (clear bars) after challenged for 24 h either apically or apically and basolaterally at the same time. The basal medium (checked bars) only showed a significant increase after the simultaneous challenge from both sides. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The cell viability (figure 7.10 A) of C38 mono-cultures was significantly decreased to a similar degree after all three challenges. The reading of FU from the control TWs was 3766.4 ± 79.97 FU compared to 2838.69 ± 232.14 FU when challenged apically, to 2261.26 ± 72.95 FU when challenged basolaterally and to 2390.87 ± 519.34 FU when challenged from both sides. TER (figure 7.10 B) of C38 mono-culture was measured before (clear bars) and after (striped bars) this 24 h exposure to live *P. aeruginosa* but was found to be stable without showing any significant changes, compared to the control, which showed a TER of 51.85 ± 3.79 Ω x cm^2 beforehand and of 52.98 ± 1.56 Ω x cm^2 afterwards. After collecting all supernatants and analysing these for their IL-8 concentration (figure 7.10 C) it was observed that IL-8 was

significantly increased in the apical supernatants (clear bars) after C38 were challenged apically or apically and basolaterally at the same time. The IL-8 concentration of the control TWs was 63.56 ± 9.36 pg/ml in the apical supernatant and 48.73 ± 4.70 pg/ml in the basal medium of the mono-culture. When C38 were challenged apically this increased to 261.81 ± 49.89 pg/ml IL-8 in the apical supernatant and increased non significantly to 107.68 ± 9.54 pg/ml in the basal medium. Both samples following the simultaneous challenge were significantly different from control and increased to 274.92 ± 57.14 pg/ml IL-8 in the apical supernatant and to 137.61 ± 26.16 pg/ml IL-8 basolaterally.

7.4.2.3 Exposure of IB3-1 mono-cultures at ALI to live bacteria

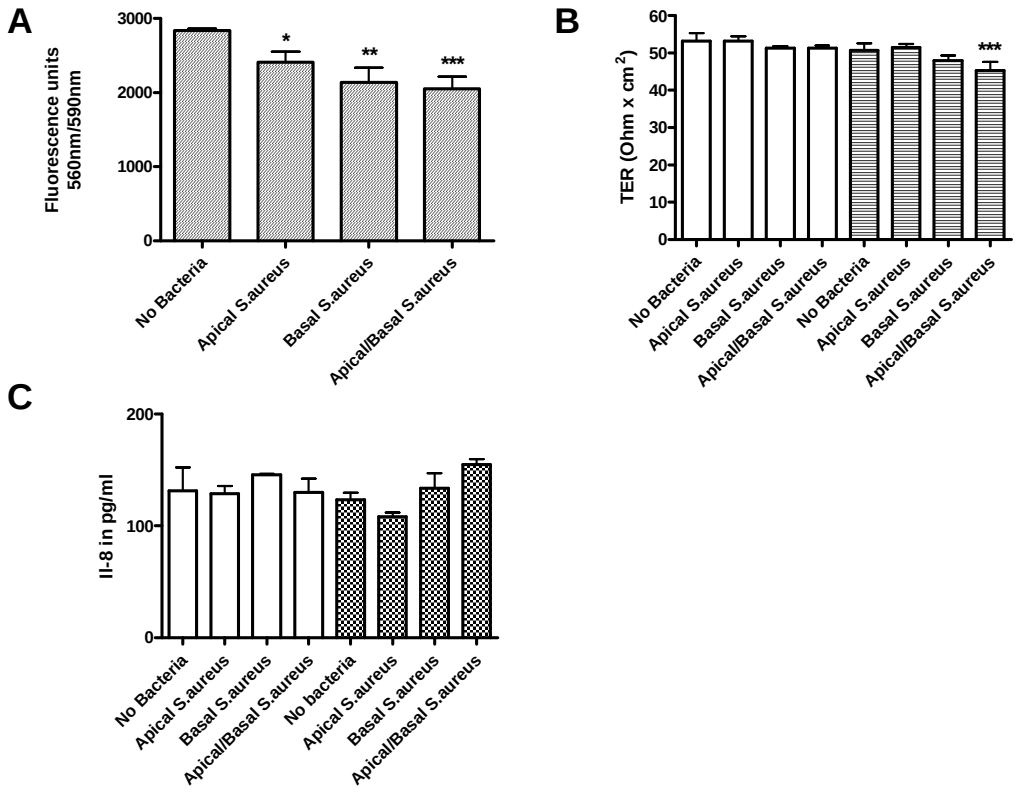

Figure 7.11 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 grown at ALI after exposure to *S. aureus*
IB3-1 were at ALI for 14 days before they were challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides at the same time (apical/basal). Control TWs with IB3-1 on their own without bacteria were treated the same way as all other TWs with medium applied apically throughout the experiment. Cell viability was significantly decreased for all three exposures when compared to the control. TER after the exposure to live *S. aureus* (striped bars) was only significantly different when IB3-1 were exposed to live *S. aureus* simultaneously from the apical and basal side of the TW compared to TER measured before (clear bars). IL-8 concentration was not affected by this bacterium and did not significantly change. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The effects of *S. aureus,* in its intact state on IB3-1, on cell viability (figure 7.11 A), TER (B) and IL-8 secretion (C) were analysed. Cell viability after exposure to live *S .aureus* was significantly decreased irrespective of the direction of challenge. FU in the cell viability assay for the control TWs of IB3-1 was 2840.95 ± 24.73 FU, which decreased to 2411.63 ± 141.57 FU when challenged apically, to 2135.25 ± 203.21 FU when challenged on the basal side and to 2050.37 ± 165.8 when live *S. aureus* was applied simultaneously on both sides. TER (B) was measured before (clear bars) and after (striped bars) the challenges with live *S. aureus*. Only when live bacteria were applied apically and basolaterally at the same time was a significant decrease observed. Before the challenge these TWs showed a TER of 51.26 ± 0.76 Ω x cm^2, which was only 45.4 ± 2.26 Ω x cm^2 after the 24 h incubation with live *S. aureus*. IL-8 secretion (7.11 C)of this mono-culture was not induced by any of these three different ways of exposure. The IL-8 concentration found in control TWs was 131.46 ± 20.84 pg/ml on the apical side and 123.40 ± 6.35 pg/ml in the basolateral medium.

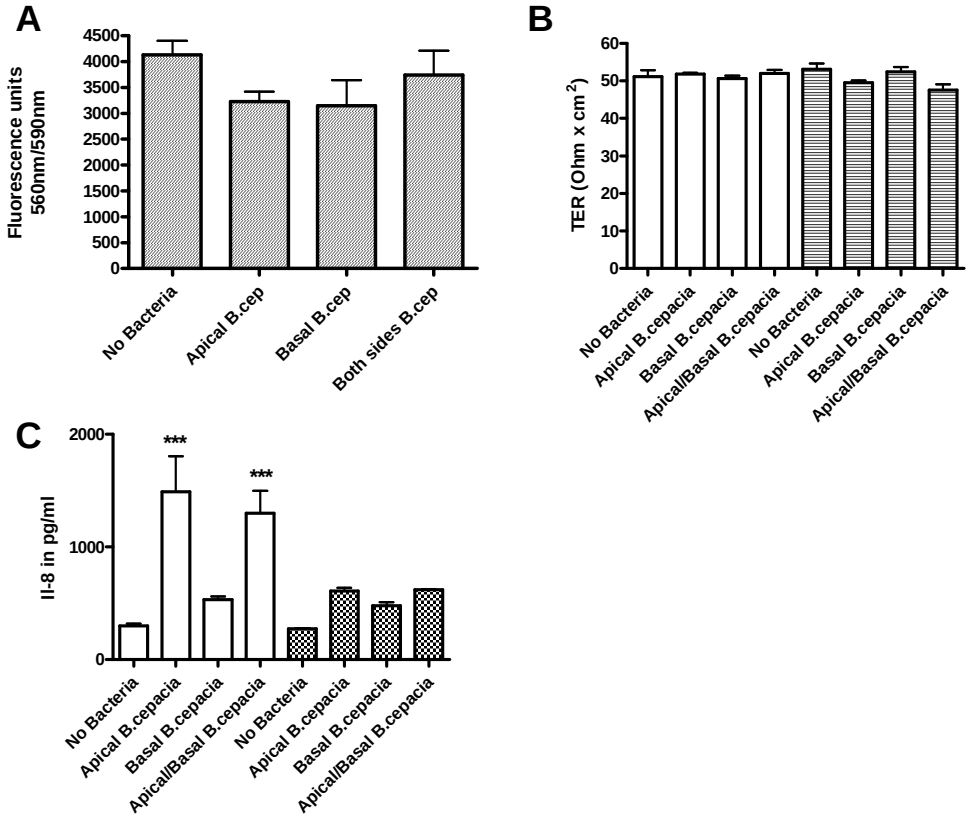

Figure 7.12 Cell viability (A), TER (B) and IL-8 release (C) after exposure of IB3-1 grown at ALI to live *B. cepacia*

IB3-1 mono-cultures were grown at ALI for 14 days before they were challenged with live *B. cepacia*, which was applied either apically, basolaterally or from both sides at the same time. IB3-1 on their own without bacteria were treated the same way as all other TWs and served as control. In this case cell viability was not significantly changed by the exposure to the bacteria. TER was found to stable for all three challenges after 24 h incubation (striped bars) when compared to TER of control TWs before (clear bars) and after (striped bars) the 24 h challenge. IL-8 concentrations observed in apical supernatants (clear bars) were significantly increased after live *B. cepacia* was applied apically or simultaneously on both sides. The basal medium collected (checked bars) did not show any significant changes. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The cell viability (figure 7.11 A) of IB3-1 mono-cultures at ALI did not show any significant changes after 24 h exposure to *B .cepacia* . A slight decrease in cell viability was seen for apically or basolaterally challenged IB3-1 but this was not significantly different when compared to the baseline fluorescence of the control, which was 4127.91 ± 277.62 FU. TER (figure 7.11 B) of all challenged TWs compared to the control was found to be stable and was not significantly changed. The control TWs showed a TER of 51.19 ± 1.75 Ω x cm^2 before the challenge and 53.13 ± 1.52 Ω x cm^2 after the challenge. Secretion of IL-8 (figure 7.11 C) was measured and found to be significantly increased after 24 h exposure to live *B. cepacia* when challenged apically or when

challenged apically and basolaterally at the same time. For the control mono-cultures 297.74 ± 21.11 pg/ml of secreted IL-8 into was found in the apical (clear bars) supernatant and 272.49 ± 6.00 pg/ml were found in the basolateral compartment (checked bars). This was significantly increased in the apical supernatants of apically challenged (1491.15 ± 314.93 pg/ml) and simultaneously challenged (1301.73 ± 196.79) IB3-1 mono-culture TWs. This increase was not apparent in the basolateral compartments of these two challenges.

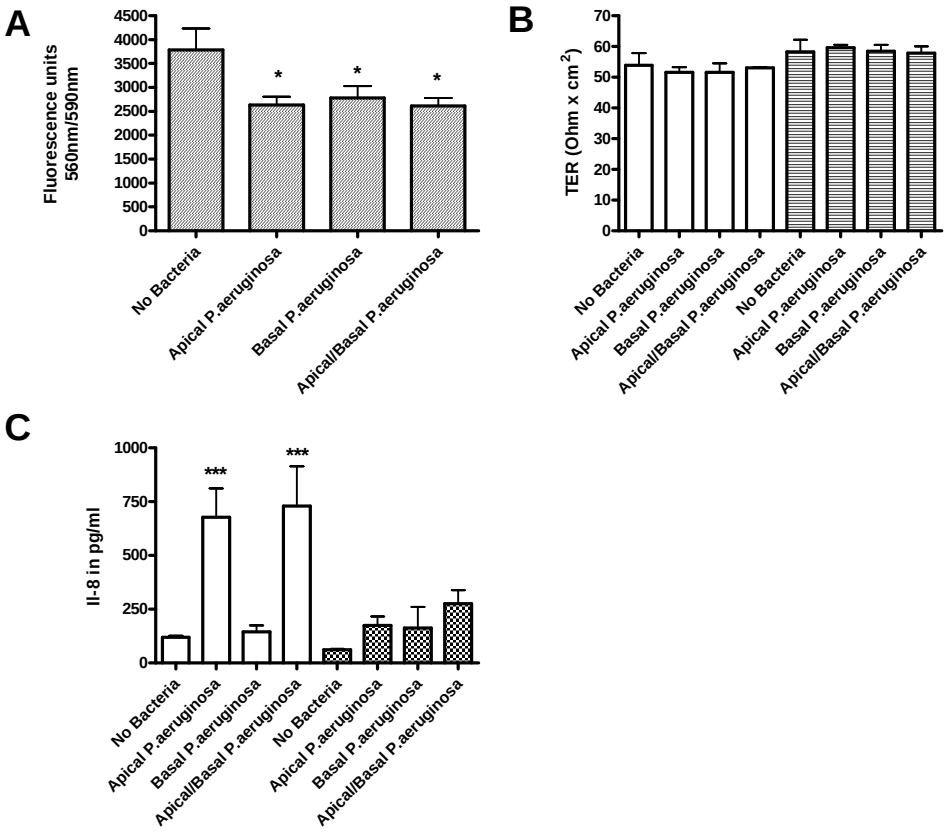

Figure 7.13 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 mono-culture after exposure to live *P. aeruginosa*

IB3-1 mono-cultures were maintained at ALI for 14 days before these cells were exposed to live *P. aeruginosa*, either apically, basally or simultaneously from both sides. Cell viability after 24 h exposure to live bacteria was significantly decreased for all three challenges compared to the control TWs, which were handled the same but without the exposure to bacteria. TER was measured before (clear bars) and after (striped bars) the experiment but no significant difference was observed after 24 h incubation with live *P. aeruginosa*. IL-8 release of IB3-1 mono-culture, however, was significantly increased by live bacteria applied apically and also when applied simultaneously on both sides. This was seen in the apical supernatants (clear bars), whereas it was not seen in the basal medium samples (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Cell viability (figure 7.13 A) of IB3-1 mono-culture was significantly affected by the exposure to live *P. aeruginosa*, when live bacteria were applied either apically, basolaterally or from both sides simultaneously. The fluorescent signal measured for the control TWs (3780.50 ± 452.94 FU) was decreased to 2635.98 ± 297.41 FU after the apical challenge. The basolateral challenge caused a decrease in cell viability to 2780.24 ± 420.81 FU and when challenged from both sides the fluorescence measured was only 2610.75 ± 286.50 FU. Despite the cell viability being decreased, unexpectedly the TER was not affected at all by this 24 h exposure to live *P. aeruginosa,* when comparing challenged TWs to the control ones. Control TWs had a TER of 53.86 ± 3.94 Ω x cm^2 before the challenge and one of 58.19 ± 3.98 Ω x cm^2. IL-8 concentrations (figure 7.13 C), however, increased from 119.83 ± 6.25 pg/ml IL-8 in the apical supernatant of the control TWs to 676.33 ± 134.97 pg/ml when challenged apically and to 728.35 ± 185.73 pg/ml when the cells are exposed to bacteria on both sides. No significant differences were observed for the basal medium samples.

7.4.2.4 Exposure of Calu-3 mono-cultures at ALI to live bacteria

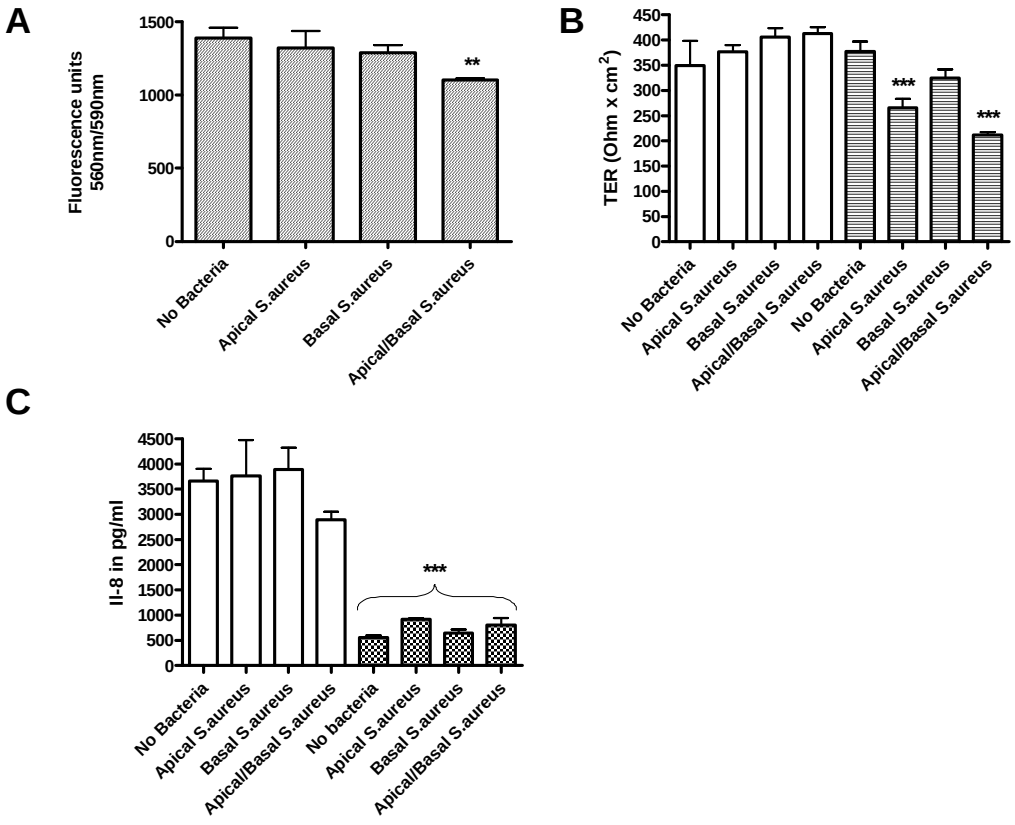

Figure 7.14 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 at ALI after exposure to *S. aureus*
Calu-3 mono-culture at ALI were challenged with live *S. aureus,* either apically, basolaterally or both at the same time after 14 days of culture. Cell viability was significantly reduced when challenged from from both sides simultaneously with live bacteria. TER was monitored before and after the challenges and was significantly decreased after *S. aureus* was applied on the apical side and when applied on both sides for 24 h incubation. IL-8 release was not increased by these exposures to live *S. aureus* in any of the apical samples (clear bars) or in any of the basal media samples (checked bars). Overall, the IL-8 concentration was significantly lower in all basal samples when compared to their corresponding apical samples. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3 mono-culture at ALI was challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides at the same time. Cell viability (figure 7.14 A) was only affected when live bacteria were applied from both sides and the FU for cell viability was reduced to 1102.64 ± 12.54 FU compared to 1390.49 ± 70.48 FU in the control TWs. TER (B) of TWs that were challenged apically was 376.75 ± 12.88 Ω x cm^2 before and was only 265.25 ± 18.1 Ω x cm^2 after. When Calu-3 mono-cultures were challenged from both sides simultaneously the TER was even lower with 211.6 ± 5.28 Ω x cm^2 compared to the TER of these TWs before, which was 412.87 ± 12.78 Ω x cm^2. IL-8 release (figure 7.13 C) of these mono-cultures was not induced by

exposure to live *S. aureus*. The concentration found in the apical supernatant (clear bars) of the control was 3660.04 ± 239.97 pg/ml and it was 551.55 ± 55.22 pg/ml in the basolateral medium (checked bars) analysed, which was significantly lower in the basal compartment. The same significant difference was observed for all other TWs as well. Basal IL-8 levels were consistently significantly lower than in the corresponding apical supernatant.

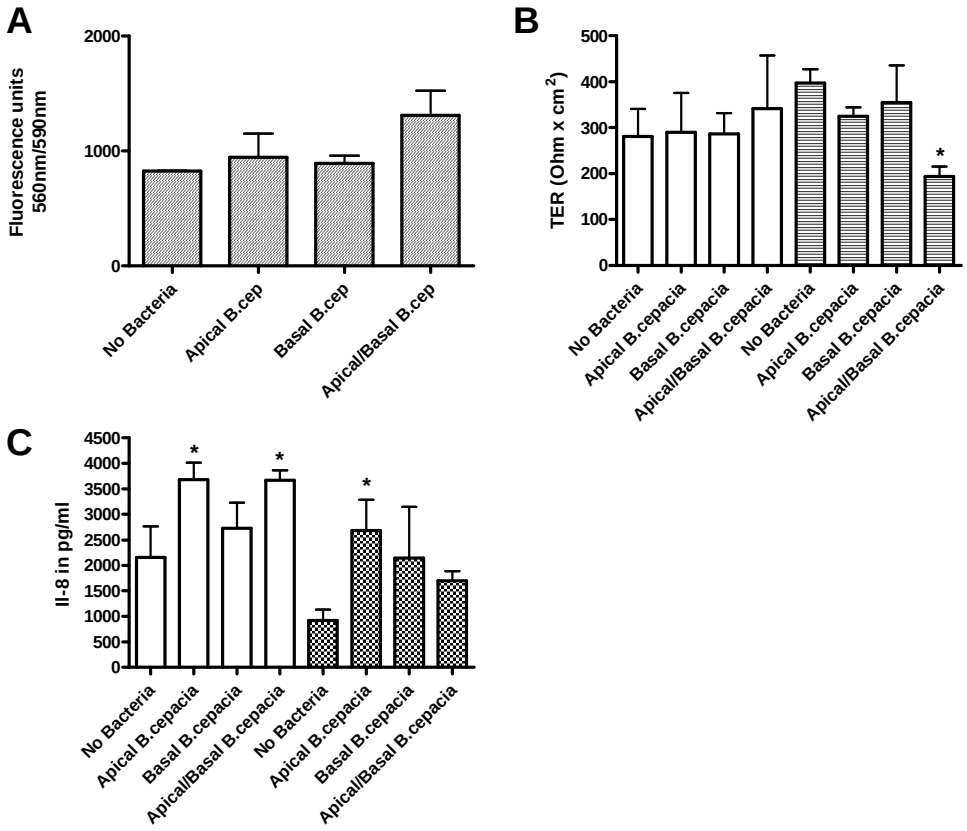

Figure 7.15 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 after exposure to live *B. cepacia*
Calu-3 mono-cultures at ALI were challenged with live *B. cepacia,* either apically, basolaterally or both at the same time after 14 days of culture at ALI. Cell viability of this mono-culture was not significantly changed throughout this bacterial challenge. TER was measured before (clear bars) and after (striped bars) the challenges and was only significantly decreased after simultaneous exposure to live *B. cepacia* on the apical side as well as on the basal side. IL-8 concentration in the apical supernatants (clear bars) was only significantly increased when challenged apically or simultaneously on both sides. The basolateral samples (checked bars) only showed a significant increase following apical challenge. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Then Calu-3 mono cultures were challenged apically, basolaterally and simultaneously on both sides with live *B. cepacia*. The cell viability (figure 7.15 A) of these mono-cultures was not changed by the exposure to live *B. cepacia* for a 24 h period and the baseline fluorescence of

control TWs was 825.33 ± 1.71 FU. The transepithelial electrical resistance was measured before (clear bars) and straight after (striped bars) the incubation with bacteria and apart from the simultaneous challenge on apical and basal side, no changes were observed. Before the exposure the TER (figure 7.15 B) of these TWs was 341.37 ± 115.5 Ω x cm^2, whereas afterwards it was 193.34 ± 21.92 Ω x cm^2. IL-8 secretion (figure7.15C) of these mono-cultures was also measured after 24 h exposure to live *B .cepacia* and was significantly increased to 3685.07 ± 325.26 pg/ml in the apical supernatant (clear bars), when the bacteria were applied to this side as well compared to 2160.67 ± 603.97 pg/ml in the apical control supernatant. The IL-8 content of the basal medium (checked bars) from apically challenged Calu-3 was also significantly increased (2683.25 ± 604.83 pg/ml) by 2.9-fold compared to the control (917.54 ± 211.45 pg/ml). When Calu-3 were challenged from both sides no significant change was observed for the IL-8 concentration in the basal medium.

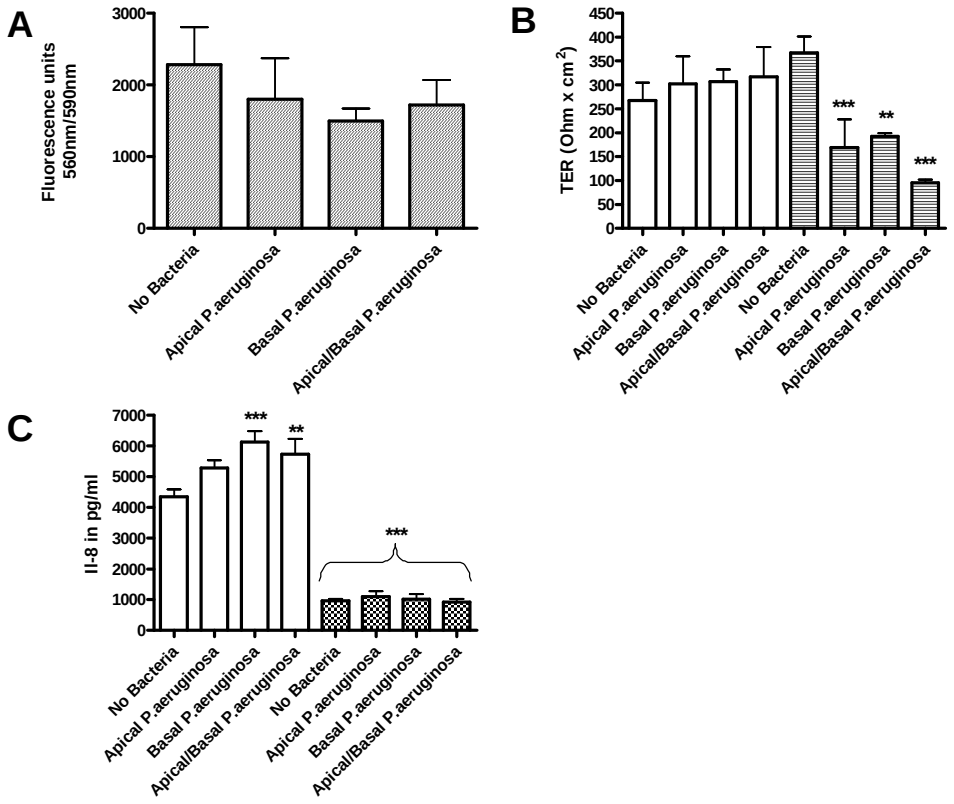

Figure 7.16 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 mono-culture after exposure to live *P. aeruginosa*

Calu-3 mono-cultures at ALI were challenged with live *P. aeruginosa,* either apically, basolaterally or both at the same time after 14 days of culture at ALI. Cell viability was not significantly decreased after any of these challenges. TER was measured before (clear bars) and after (striped bars) the challenges and was significantly decreased after all three different exposures to live *P. aeruginosa*. IL-8 concentration in the apical supernatants (clear bars) were increased significantly after the basal as well as the apical/basal challenge and in the basolateral samples (checked bars) did not show any significant changes for these challenges. The basolateral sample are all significant lower in IL-8 compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

Calu-3 mono-cultures were exposed to live *P. aeruginosa*, which was applied in three different ways. Either *P. aeruginosa* was added to the apical side of the mono-culture, to the basal medium or the bacteria were added in both compartments at the same time. After a 24 h incubation with the bacteria, cell viability (figure 7.16 A) of Calu-3 was assessed and found to be stable throughout the experiment. The baseline fluorescence that was measured for the control TWs was 2284.31 ± 519.29 FU. In contrast the electrical resistance was measured in TER (B) and was significantly decreased in all three occasions. The control TWs TER was measured and was 267.53 ± 37.64 Ω x cm^2 before (clear bars) and was 367.29 ± 33.62 Ω x cm^2 after (striped bars) the 24 h incubation. Following the apical challenge the TER fell from 302.03 ± 57.36 Ω x cm^2 to

169.32 ± 58.6 Ω x cm^2, for the basolateral challenge it fell from 306.53 ± 25.97 Ω x cm^2 to 192.57 ± 6.53 Ω x cm^2 and when challenged simultaneously the TER of Calu-3 mono-culture fell from 316.58 ± 62.72 Ω x cm^2 to 95.40 ± 6.31 Ω x cm^2. IL-8 concentration (C) of this mono-culture was observed and only when challenged basally or from both sides at the same time, a significant increase was seen. The basally challenged apical supernatant (clear bars) had an IL-8 concentration of 6121.55 ± 363.81 pg/ml compared to 4346.8 ± 232.60 pg/ml found in the control apical supernatant. When challenged from both sides an IL-8 concentration of 5724.85 ± 880.47 pg/ml was reached in the apical supernatant. The basal medium samples (checked bars) show the same trend of increase as the apical samples but were not significant.

7.4.2.5 Exposure of HPF-C38 co-cultures grown at ALI to live bacteria

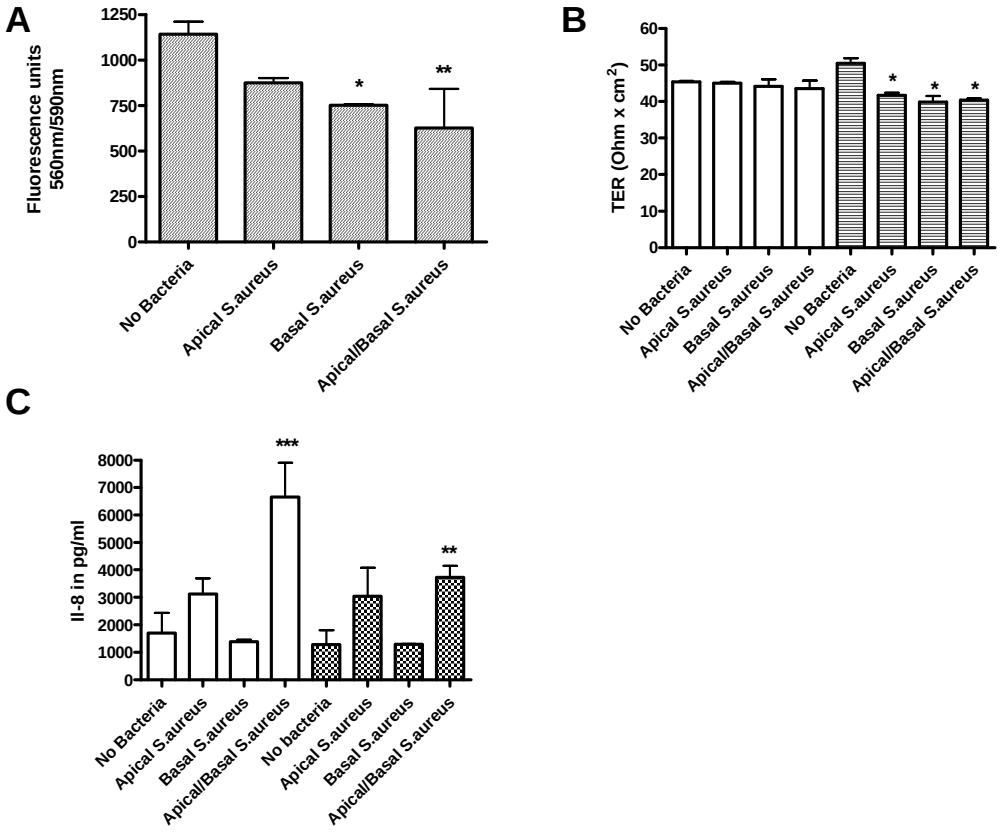

Figure 7.17 Cell viability (A), TER (B) and IL-8 release (C) after exposure to *S. aureus* of HPF-C38 grown at ALI
HPF-C38 were grown on TWs at ALI for 14 days before they were challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides simultaneously. HPF-C38 without bacteria, which were treated the same way throughout the experiment with medium only served as control. Cell viability was significantly decreased compared to the control, when this co-culture was challenged basolaterally or when challenged from both sides at the same time. The measured TER after the challenges (striped bars) was significantly lower in all bacterial challenged TWs compared their corresponding values before (clear bars). IL-8 secretion was significantly increased by simultaneous treatment with live *S. aureus* from apical and basal side. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Co-cultures of HPF and C38 were grown at ALI for 14 days before the challenges were carried out. Cell viability (figure 7.17 A) was assessed after the challenges with live *S. aureus* and was in this case significantly affected when challenged basolaterally (751.71 ± 5.88 FU) or from the apical and basal side at the same time (626.56 ± 214.59 FU) when compared to the control TWs (1143 ± 67.54 FU). The average TER (figure 7.17 B) for the control TWs measured after (striped bars) the 24 h was 50.45 ± 1.4 Ω x cm^2 and was decreased significantly after all three challenges with live *S. aureus*. After 24 h apical exposure to live *S. aureus* the TER was 41.7 ± 0.61 Ω x cm^2,

after basal exposure it was 39.86 ± 1.6 Ω x cm^2 and when challenged from both sides the TER fell to 40.38 ± 0.48 Ω x cm^2 compared to their corresponding measurement before the challenges. IL-8 concentrations (figure 7.12 C) that were observed were only significantly increased when live *S. aureus* was applied to both sides of the co-culture simultaneously. IL-8 secretion at baseline was 1708.2 ± 730.8 pg/ml in the apical medium (clear bars) and 1281.94 ± 526.3 pg/ml in the basal media. This increased to 6655.4 ± 1242.29 pg/ml in the apical supernatant and to 3728.8 ± 430.9 pg/ml in the basal medium collected (checked bars).

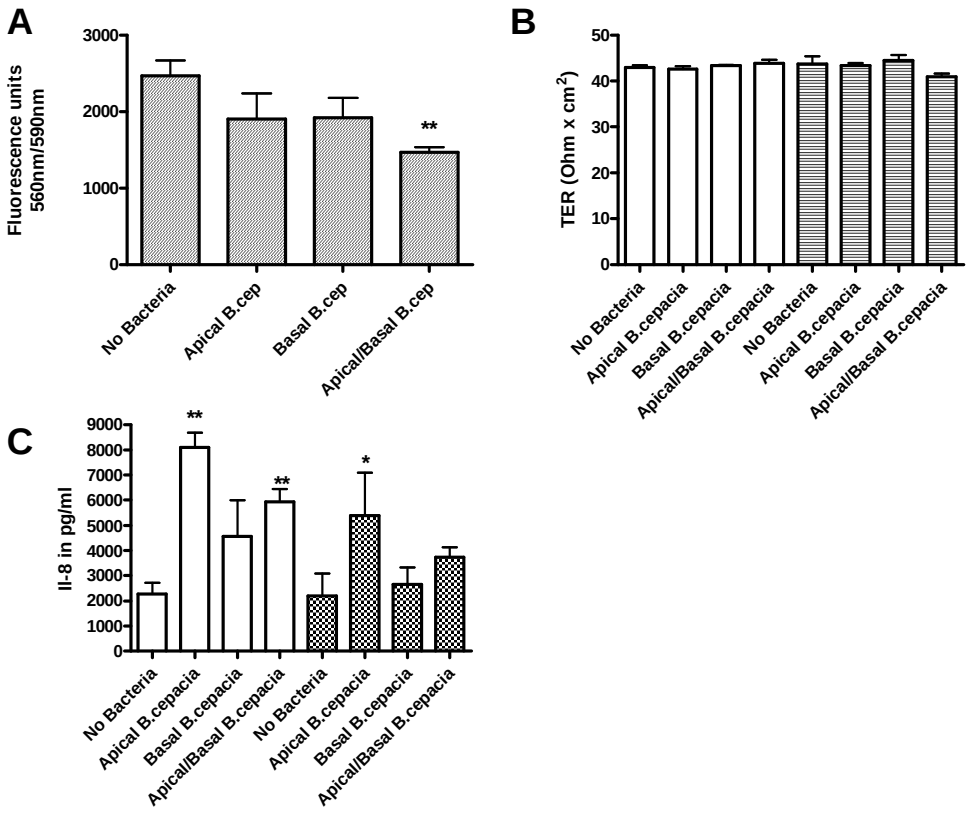

Figure 7.18 Cell viability (A), TER (B) and IL-8 release (C) after exposure to live *B. cepacia* of HPF-C38 co-culture grown at ALI

HPF-C38 co-culture was challenged with live *B. cepacia*, which were applied to either the apical side of the culture, to the basolateral side or to both at the same time. HPF-C38 on their own without bacteria were treated the same way as all other TWs and served as control. In this case cell viability was significantly changed by the exposure to live *B. cepacia*, when the bacteria were applied on both sides of the co-culture. However, the TER measured was found to be stable for all three challenges after (striped bars) 24 h incubation when compared to TER of control TWs before (clear bars) and after (striped bars) the 24 h challenge. IL-8 concentrations observed in apical supernatants (clear bars) were significantly increased after live *B. cepacia* was applied apically as well as when applied on both sides. This was only reflected in the basal medium for the apical challenge but not when challenged on both sides (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The only change in cell viability (figure 7.18 A) observed for HPF-C38 was a significantly decrease when this co-culture was challenged simultaneously from the apical as well as the basolateral side. Cell viability of control TWs was 2468.3 ± 199.8 FU compared to only 1465.8 ± 72.66 FU after they were challenged with live *B. cepacia*. The TER (figure 7.18 B) was stable throughout these experiments and was not significantly affected for this period of bacterial exposure, when compared to the control cultures, which had a TER of 42.94 ± 0.55 Ω x cm^2 before the exposure to live *B. cepacia* and a TER of 43.75 ± 1.61 Ω x cm^2. This was different from the analysis of IL-8 concentrations (figure 7.18 C). Here, a significant increase was observed when C38-HPF co-cultures were challenged from the apical side and IL-8 concentration (figure 7.18 C) increased to 8095.19 ± 588.34 pg/ml in the apical supernatant (clear bars) compared to 2271.92 ± 439.19 pg/ml of the apical supernatant of the control TWs. When the co-culture was challenged from both sides simultaneously IL-8 concentration was significantly increased to 5920.66 ± 518.52 pg/ml compared to the control for the apical supernatants. After the basolateral samples were analysed, a significant increase was only observed after apical challenge with live *B. cepacia*. The concentration of IL-8 in the basal medium of control TWs was 2196.14 ± 880.8 pg/ml and increased to 5385.95 ± 1706.45 pg/ml after the challenge.

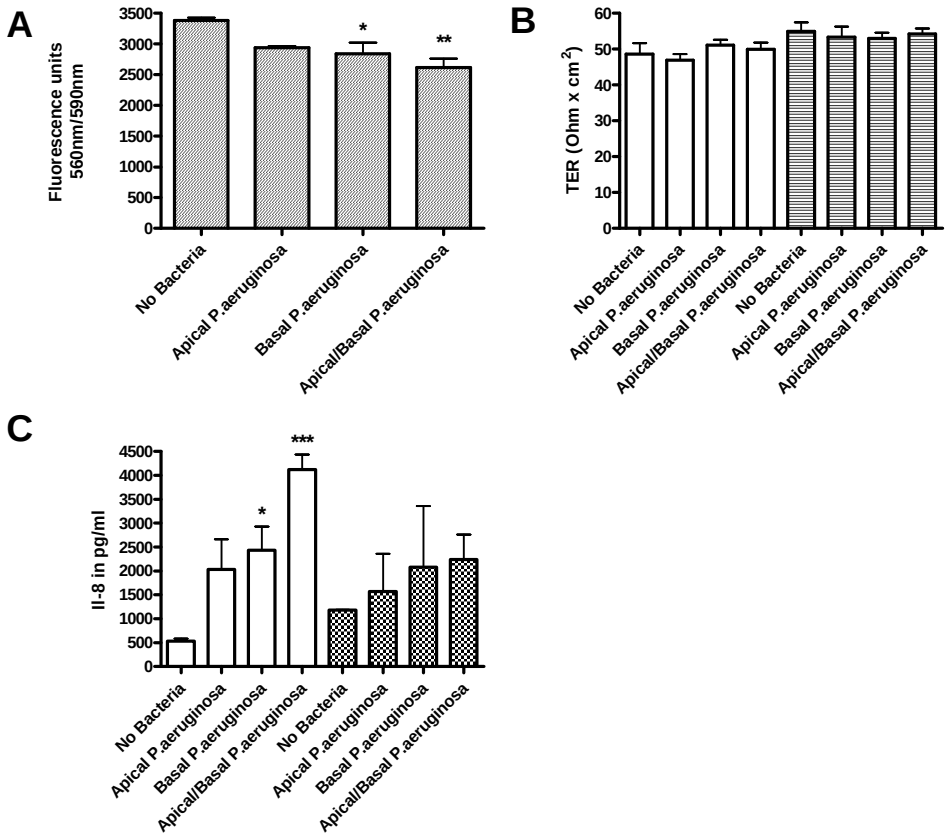

Figure 7.19 Cell viability (A), TER (B) and IL-8 release (C) after exposure to live *P. aeruginosa* of HPF-C38 grown at ALI
HPF-C38 were grown on TWs at ALI for 14 days before they were challenged with live *P. aeruginosa*, which was applied either apically, basolaterally or from both sides simultaneously. HPF-C38 without bacteria, which were treated the same way throughout the experiment with medium only, served as control. Cell viability was significantly decreased compared to the control, when this co-culture was challenged basolaterally or when challenged from both sides at the same time. The measured TER before (clear bars) and after the challenges (striped bars) was not changed by this 24 h incubation with live *P. aeruginosa*. IL-8 secretion, however, was significantly increased by basolateral and simultaneous treatment with live *P. aeruginosa* from apical and basal side and this was only observed in the apical (clear bars) supernatants. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

HPF-C38 co-culture was also exposed to live *P. aeruginosa*. The cell viability (figure 7.19 A) was decreased significantly after the co-culture was exposed to *P. aeruginosa* on the basal side and when exposed to the bacteria on both sides. Cell viability of control TWs gave a reading of 3379.93 ± 46.99 FU, which was only 2838.16 ± 313.03 FU after the basolateral challenge and only 2614.56 ± 250.95 FU after the simultaneous challenge from both sides. The electrical resistance properties, which were measured as TER (figure 7.19 B) were not affected by these challenges after 24 h. TER measured for the control cultures were 48.58 ± 3.05 Ω x cm^2before (clear bars) the challenge and it was 54.85 ± 2.59 Ω x cm^2 after the challenge (striped bars). IL-8

secretion (figure 7.19 C) of this co-culture significantly increased after TWs were exposed to basolateral bacteria and increased from 535.25 ± 51.28 pg/ml in the apical supernatant (clear bars) of the control TWs to 2432.04 ± 495.78 pg/ml and even higher to 4118.35 ± 544.57 pg/ml when these co-cultures were challenged from both sides at the same time. The same trend of increasing IL-8 concentration was seen in the basal medium samples (checked bars) but was not found to be significant.

7.4.2.6 Exposure of HPF-IB3-1 co-cultures grown at ALI to live bacteria

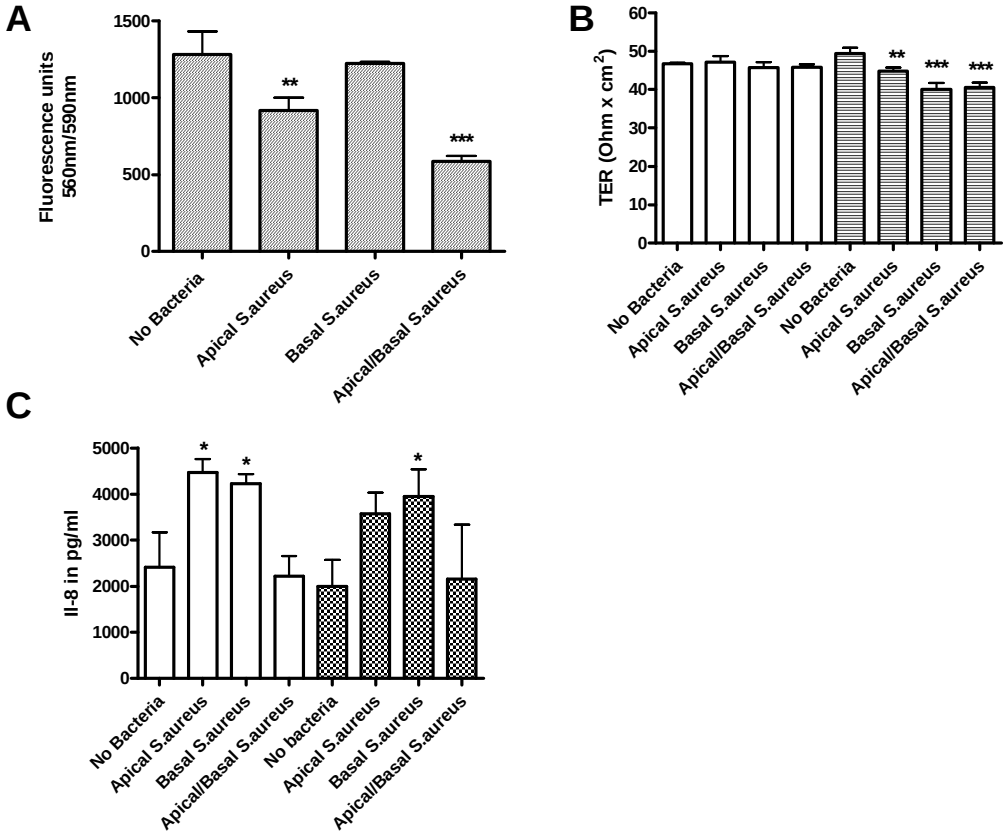

Figure 7.20 Cell viability (A), TER (B) and IL-8 release (C) after exposure to live *S. aureus* of HPF-IB3-1 grown at ALI

HPF-IB3-1 co-cultures were at ALI for 14 days before they were challenged with live *S. aureus*, which was applied either apically, basolaterally or from both sides at the same time. HPF-IB3-1 on their own without bacteria were treated the same way as all other TWs and served as control. In this case cell viability was significantly decreased when the co-culture system was challenged apically or simultaneously from both sides with live *S. aureus*. TER was found to be significantly decreased for all three challenges after 24 h incubation (striped bars) when compared to TER of control after the 24 h challenge. IL-8 concentrations observed in apical supernatants (clear bars) were significantly increased after live *S. aureus* was applied apically or basolaterally. The basal medium collected (checked bars) showed a significant increase only for basal application of *S. aureus*. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

HPF-IB3-1 co-cultures were exposed to live *S. aureus* after being grown for 14 days at ALI. *S. aureus* was applied apically, basolaterally or on both sides at the same time. The cell viability (figure 7.20 A) was observed after 24 h exposure to the bacteria and was significantly decreased for apically treated cells and for simultaneous treatment from both sides compared to untreated cells. The baseline FU for the control was 1282.48 ± 150.1 FU compared to the reduced value for apically challenged cells, which was 917.87 ± 84.58 FU and TWs challenged on both sides, which was only 586.77 ± 34.44 FU. The integrity of the epithelial cell layer was assessed by TER (figure 7.20 B) and this was significantly lower for all three challenges compared to the control TWs. After the 24 h challenge the control's TER was 49.42 ± 1.53 Ω x cm^2 compared to 44.84 ± 0.92 Ω x cm^2 when challenged apically, 40.0 ± 1.65 Ω x $cm^2$when challenged basolaterally and compared to 40.55 ± 1.3 Ω x cm^2 when live *S. aureus* was applied on both sides. The concentration of IL-8 (figure 7.20 C) in the apical supernatants (clear bars) was significantly increased for two occasions. The first significant increase compared to the control TWs (2407.27 ± 755.82 pg/ml) was noted, when HPF-IB3-1 were challenged apically (4469 ± 290.8 pg/ml) and the other one when these cells were challenged basolaterally (4224.73 ± 210.02 pg/ml), which also caused a significant increase of IL-8 in the basal medium (checked bars) collected, which had a concentration of 3945.4 ± 590.37 pg/ml compared to (1995.18 ± 574.49 pg/ml) basal medium of the control TWs.

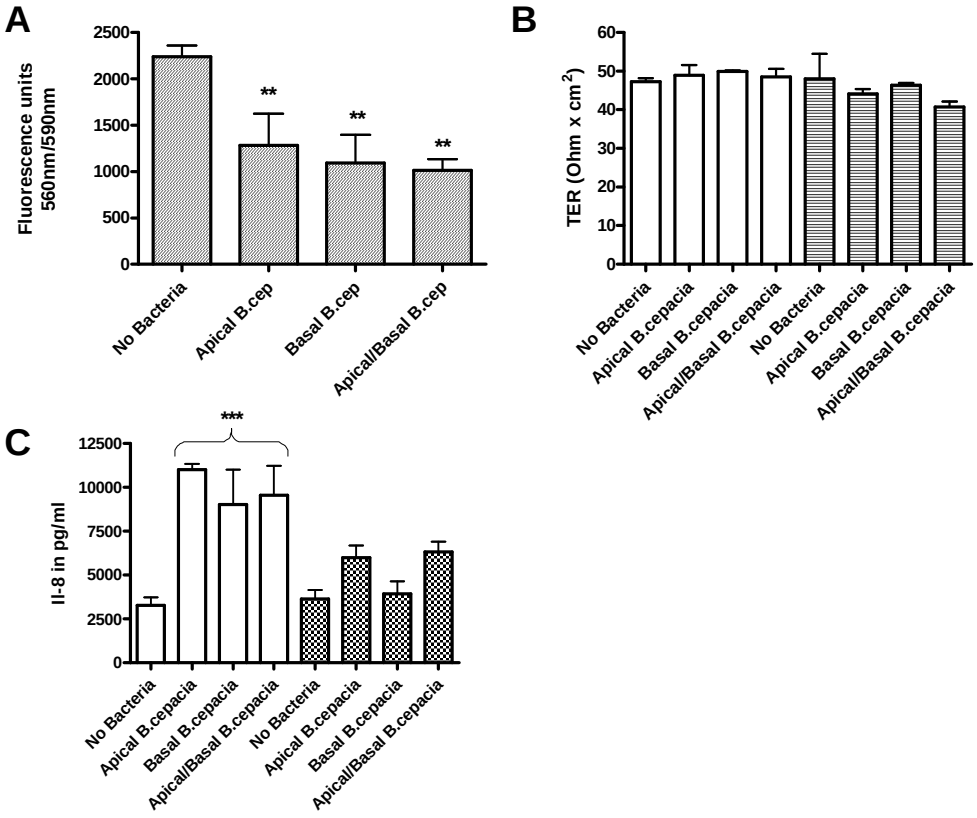

Figure 7.21 Cell viability (A), TER (B) and IL-8 release (C) after exposure to live *B. cepacia* of HPF-IB3-1 grown at ALI

HPF-IB3-1 co-cultures were at ALI for 14 days before they were challenged with live *B. cepacia*, which was applied either apically, basolaterally or from both simultaneously. HPF-IB3-1 on their own without bacteria were treated the same way as all other TWs and served as control. In this case cell viability was significantly decreased after all three challenges with live *B. cepacia* for 24 h. TER was found to be stable for all three challenges and did not change. IL-8 concentrations observed in apical supernatants (clear bars) were significantly increased after live *B. cepacia* were applied apically, basolaterally or on both sides at the same time. The IL-8 concentrations in basal media samples was not significantly changed and only showed a trend of increasing (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each).

HPF-IB3-1 co-cultures were investigated for changes in cell viability (figure 7.21 A), TER (figure 7.21 B) and IL-8 secretion (figure 7.21 C) into the medium after exposure to live *B. cepacia*, which was applied either apically, basolaterally or on both sides. Cell viability was significantly decreased in all three cases and decreased to 1280.31 ± 340.68 FU when challenged apically, to 1093.05 ± 301.41 FU when challenged basally and to 1013.08 ± 121.22 FU when exposed to live bacteria on both sides compared to 2236.16 ± 120.41 FU measured for the control TWs. Even though cell viability was significantly lower the TER was not affected by this after 24 h incubation, when comparing challenged TWs to the control ones, which had a TER of 47.23 ± 0.90 beforehand (clear bars) and a TER of 47.96 ± 6.49 afterwards (striped bars). IL-8 concentrations were significantly increased in the apical supernatants (clear bars) after all three

challenges. IL-8 concentration measured for the control TWs was 3289.55 ± 447.96 pg/ml and was in increased to 11012.96 ± 325.18 pg/ml for the apical challenge, to 9022.79 ± 1990.16 pg/ml for the basaolateral challenge and to 9545.64 ± 1680.05 pg/ml when challenged from both sides. The same trend was seen for basal medium (checked bars) analysed for IL-8 but this was not significant.

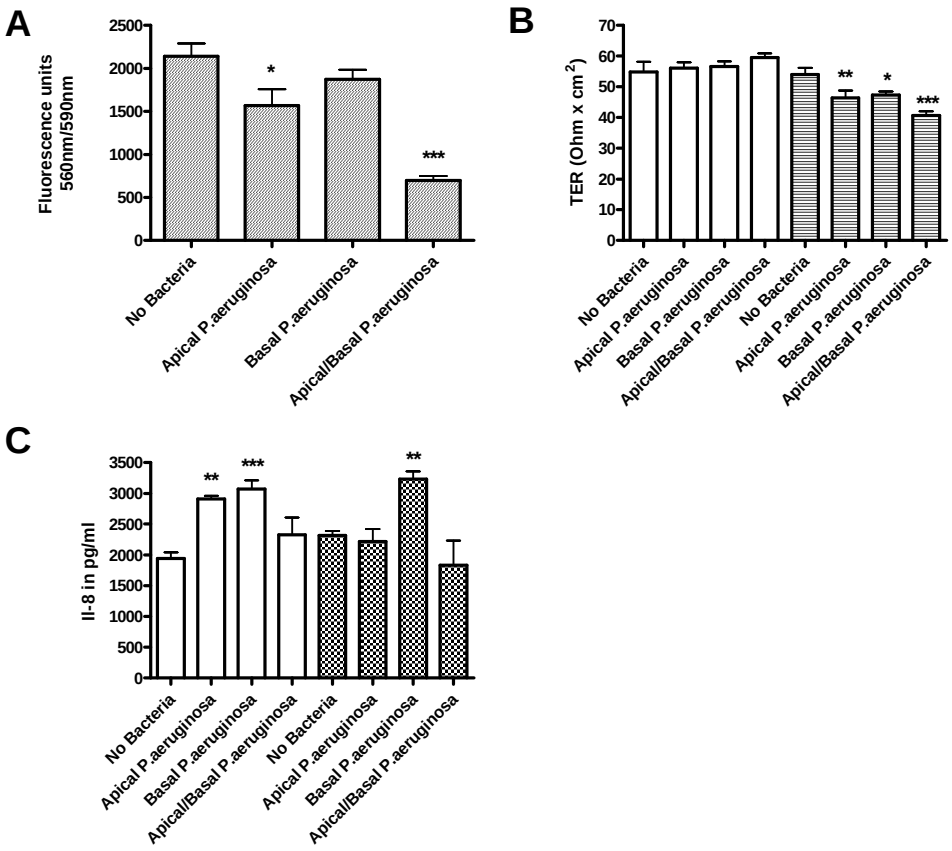

Figure 7.22 Cell viability (A), TER (B) and IL-8 release (C) after exposure to live *P. aeruginosa* of HPF-IB3-1 grown at ALI

HPF-IB3-1 co-cultures were grown at ALI for 14 days before they were challenged with live *P. aeruginosa*, which was applied either apically, basolaterally or from both sides at the same time. HPF-IB3-1 on their own without bacteria were treated the same way as all other TWs and served as control. In this case cell viability was significantly decreased when the co-culture system was challenged apically or simultaneously from both sides with live *P. aeruginosa*. TER was found to be significantly decreased for all three challenges after 24 h incubation (striped bars) when compared to their corresponding TER bar before challenge. IL-8 concentrations observed in apical supernatants (clear bars) were significantly increased after live *P. aeruginosa* was applied apically or basolaterally. The basal medium collected (checked bars) showed a significant increase in IL-8 only after basal application of *P. aeruginosa*. Data are presented as mean ± SD of 3 individual experiments (3TWs each).

HPF-IB3-1 co-cultures were challenged with live *P. aeruginosa,* which was either applied apically, basolaterally or from both sides at the same time. This 24 h incubation with live bacteria had a huge impact on cell viability (figure 7.22 A) and decreased it from 2140.0 ± 145.77 FU to 1568.00 ± 327.55 FU when challenged apically and to 697.84 ± 91.13 FU when challenged from apical and basal side at the same time. TER (figure 7.22 B) was analysed before (clear bars) and after (striped bars) these challenges and was also found to be affected as the control TWs showed a TER of 54.82 ± 3.34 Ω x cm^2 before the challenge and 54.01 ± 2.10 Ω x cm^2 after the challenge, whereas after the apical challenge it only was 46.35 ± 2.44 Ω x cm^2 (before 56.02 ± 1.91 Ω x cm^2), after the basal challenge it was only 47.34 ± 1.10 Ω x cm^2 (before 56.61 ± 1.61 Ω x cm^2) and when challenged from both sides the TER fell to 40.7 ± 1.29 Ω x cm^2 (before 59.54 ± 1.31 Ω x cm^2). IL-8 concentrations (figure 7.22 C) found in apical supernatants (clear bars) and basal medium (checked bars) were also significantly changed. 1944.97 ± 98.62 pg/ml were found in apical supernatant of the control TWs and 2317.03 ± 73.09 pg/ml was found in the basal medium. Significant increases were observed for apical supernatants of the apical challenge (2909.78 ± 48.94 pg/ml) as well as of the basolateral challenge (3071.98 ± 140.40 pg/ml). For the basal medium of these samples there was only one significant change and that was found for basolaterally challenged TWs that showed an IL-8 concentration of 3231.98 ± 126.09 pg/ml.

7.4.2.7 Exposure of HPF-Calu-3 co-cultures grown at ALI to live bacteria

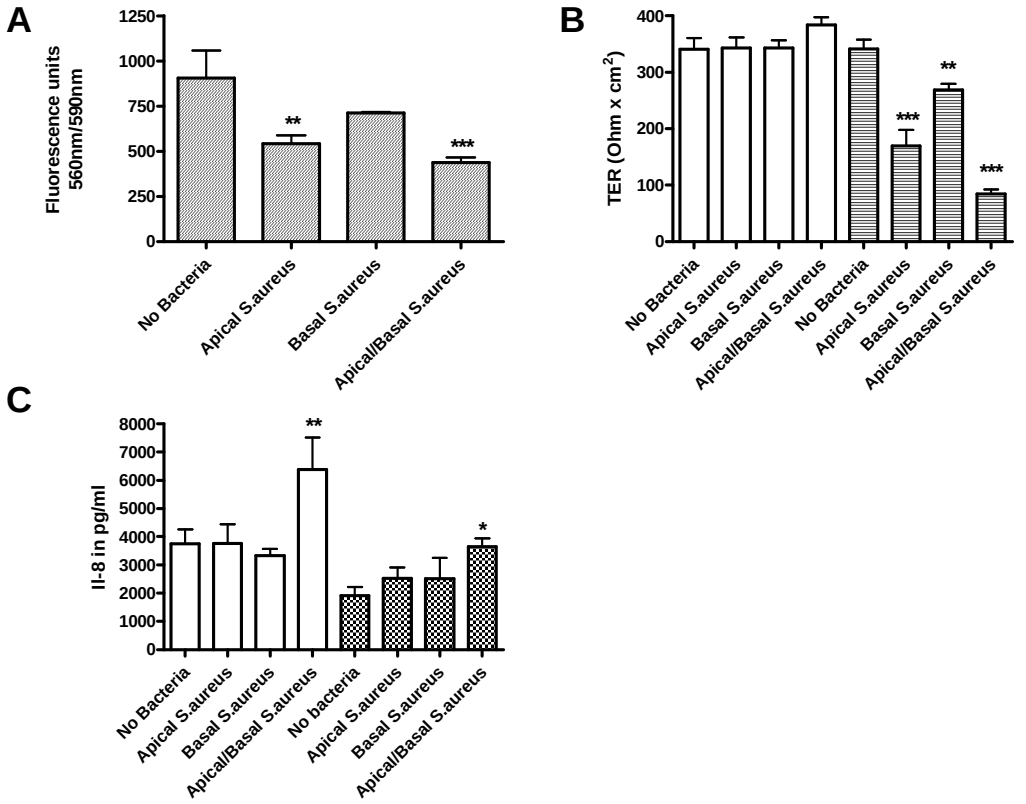

Figure 7.23 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 after exposure to live *S. aureus*
HPF-Calu-3 co-culture at ALI were challenged with live *S. aureus,* either apically, basolaterally or both at the same time after 14 days of culture. Cell viability was significantly reduced when challenged from apically or when challenged apically and basolaterally at the same time with live *S. aureus*. TER was measured before (clear bars) and after (striped bars) the challenges and was significantly decreased after all three challenges with live *S. aureus*. IL-8 release was only increased significantly when challenged simultaneously on both sides. This was found for the apical supernatant (clear bars) as well as the basal medium collected (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each).

HPF-Calu-3 co-cultures were challenged with live *S. aureus* after they were grown for 14 days at ALI. The cell viability (figure 7.23 A) was significantly decreased compared to the control TWs (904.6 ± 154.63 FU) when *S. aureus* was applied apically (541.2 ± 48.19 FU) or from apical and basal side at the same time (436.94 ± 29.5 FU). TER (figure 7.23 B) was monitored before (clear bars) and after (checked bars) the challenges and was significantly decreased for all three challenges compared to their corresponding value before (clear bars). For the apical challenge, the TER fell from 343.12 ± 18.21 Ω x cm^2 to 169.91 ± 27.64 Ω x cm^2, for the basolateral challenge it went from 343.2 ± 13.6 Ω x cm^2 down to 268.98 ± 10.77 Ω x cm^2 and when challenged from both sides at the same time it drops from 383.75 ± 13.85 Ω x cm^2 to 84.63 ± 7.75 Ω x cm^2. IL-8

(figure 7.23 C) was measured in all apically collected supernatants (clear bars) as well as in the basal media (checked bars) of each TW and significant differences were only found when this co-culture was challenged on both sides simultaneously compared to the control, which had an IL-8 concentration of 3751.72 ± 503.98 pg/ml in the apical supernatant and 1915.62 ± 291.65 pg/ml in the basal medium. This concentration increased to 6386.6 ± 1133.81 pg/ml in the apical supernatant and to 3648.55 ± 294.02 pg/ml in the basolateral medium.

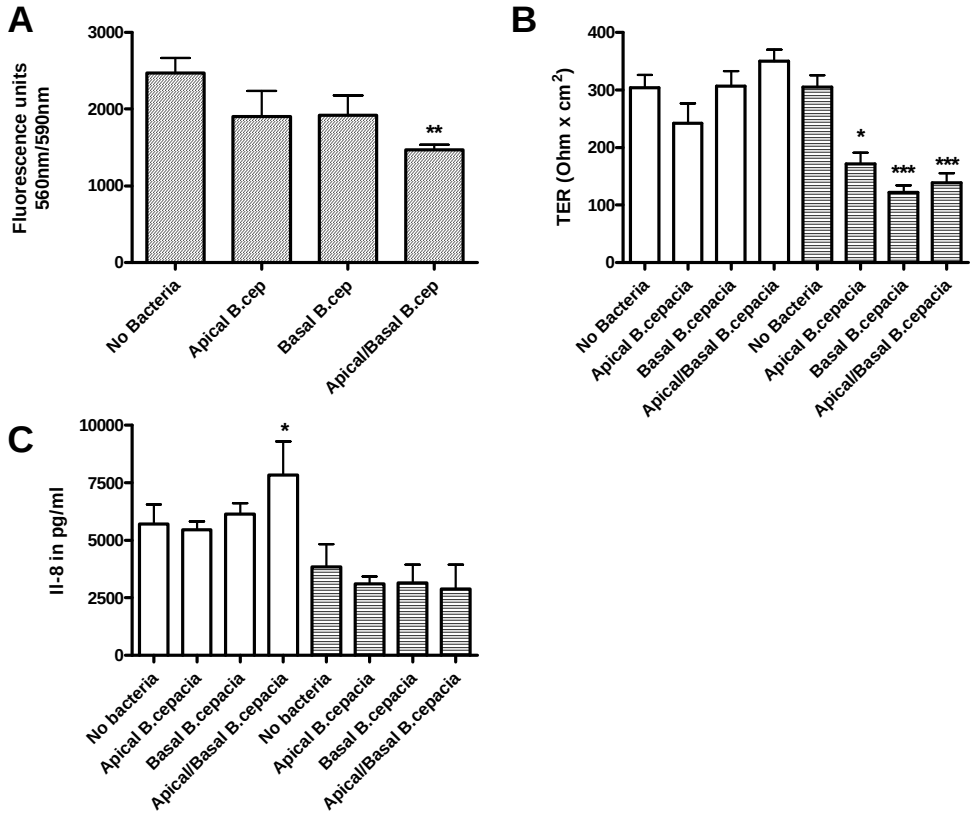

Figure 7.24 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to live *B. cepacia*

HPF-Calu-3 co-cultures at ALI were challenged with live *B. cepacia,* either apically, basolaterally or both at the same time after 14 days of culture at ALI. Cell viability of this co-culture was significantly decreased when challenged from both sides with live bacteria. TER was measured before (clear bars) and after (striped bars) the challenges and was significantly decreased after all three different exposures to live *B. cepacia*. IL-8 concentration in the apical supernatants (clear bars) was only significantly increased when challenged apically and basally at the same time. The basolateral samples (checked bars) did not show a significant increase for these challenges. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-Calu-3 cell co-culture viability (figure 7.24 A) was analysed after 24 h exposure to live *B. cepacia* and was found to be decreased significantly when challenged on both sides of the cells.

The FU of the control TWs measured were 2468.27 ± 199.8 FU and this was decreased to 1465.8 ± 72.66 FU after these co-cultures were exposed to live *B. cepacia* simultaneously on both sides. TER (figure 7.12B) was affected by all three different challenges significantly and was decreased to 171.34 ± 19.47 Ω x cm^2 (before 242.03 ± 34.65 Ω x cm^2) after the apical exposure, lowered to 122.03 ± 12.167 Ω x cm^2 (before 306.53 ± 25.97 Ω x cm^2) after the basal challenge and fell down to 138.93 ± 16.33 Ωcm^2 (before 349.91 ± 20.62 Ωcm^2) after simultaneously challenging the co-culture on both sides. Even though these two parameters showed significant changes, the IL-8 concentration shown in figure 7.24 (C) was only significantly increased in the apical compartment following simultaneous challenge of this co-culture. The control TWs showed 5709.73 ± 832.55 pg/ml in the apical supernatant (clear bars) and 3845.69 ± 991.91 pg/ml in the basolateral medium (checked bars) compared to 7836.94 ± 1456.96 pg/ml apically and 2886.73 ± 1052.6 pg/ml basally when challenged from both sides at the same time.

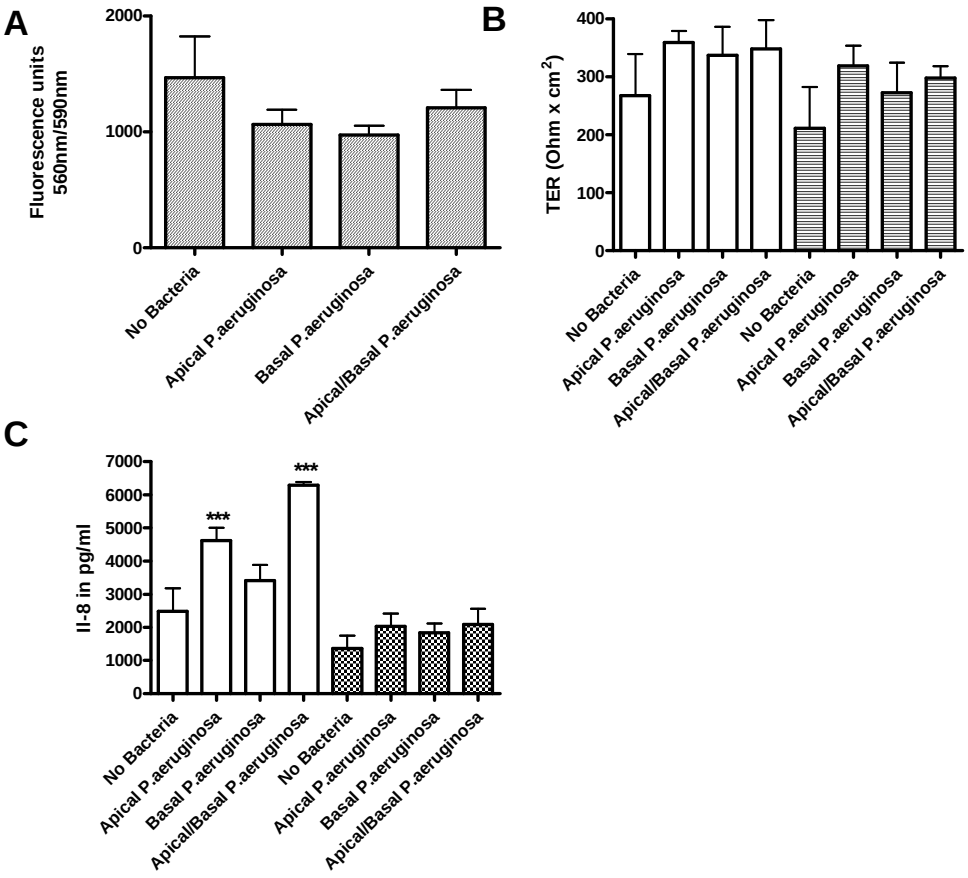

Figure 7.25 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to live *P. aeruginosa*

HPF-Calu-3 co-cultures at ALI were challenged with live *P. aeruginosa,* either apically, basolaterally or both at the same time after 14 days of culture at ALI. Cell viability of this co-culture was was not affected by any of these three challenges after 24 h. TER was measured before (clear bars) and after (striped bars) the challenges and there were no significant changes apparent after all three different exposures to live *P. aeruginosa*. IL-8 concentration in the apical supernatants (clear bars) was only significantly increased when challenged apically and simultaneously from both sides. The basolateral samples (checked bars) did not show a significant increase for these challenges. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Then HPF-Calu-3 co-cultures were challenged with live *P. aeruginosa* after they were grown for 14 days at ALI. *P. aeruginosa* was applied apically, basally or from both sides at the same time. These challenges did not significantly decrease cell viability (figure 7.25 A) after 24 h incubation with the bacteria and the baseline fluorescence of this culture was 1465.69 ± 355.19 FU. TER (figure 7.25 B) of this co-culture was not affected by the challenges at all and was stable throughout the experiment. Control co-cultures that were exposed to growth medium only had a TER of 267.41 ± 72.09 Ω x cm^2 beforehand and a TER of 210.76 ± 71.25 Ω x cm^2 afterwards. IL-8 (figure 7.25 C) concentrations, however, did increase significantly in the apical supernatants

(clear bars) after being challenged apically or from both sides. The control TWs showed an IL-8 concentration of 2488.17 ± 691.99 pg/ml compared to 4625.07 ± 374.77 pg/ml after the co-culture was challenged apically with live *P. aeruginosa* and compared to 6288.10 ± 165.71 pg/ml after they were challenged simultaneously from both sides. No significant differences could be identified for the basal media samples (checked bars) collected from the TWs.

7.5 Discussion

In this chapter submerged mono-cultures and mono-and co-cultures at ALI of HPF, C38, IB3-1 and Calu-3 were exposed to viable *S. aureus, B. cepacia* and *P. aeruginosa*, which are all CF relevant pathogens of the airways. *B. cepacia* and *P. aeruginosa* used in the present study were isolated from CF patients and were provided by Professor Peter Lambert.

Cellular responses, in terms of IL-8 release, cell viability and cell layer integrity were monitored to see what impact these CF relevant bacteria in their intact, viable form have on the established model systems. The responses of HPF mono-cultures in isolation was carried out in order to see what input this supporting cell type might have on pro-inflammatory responses to infection especially in respect of the co-culture models.

7.5.1 *S. aureus* challenge of submerged mono-cultures and mono- and co-cultures at ALI

Submerged mono-cultures of HPF, C38, IB3-1 and Calu-3 were challenged with viable *S. aureus* for 24 h. Interestingly, under these conditions only Calu-3 mono-cultures responded to live *S. aureus* by secreting IL-8 into the culture medium. The cell viability was not affected in any of these mono-cultures. These findings are completely different when cells are grown on TWs.

HPF were grown under submerged conditions on TWs and epithelial cells were grown at ALI. When grown on TWs, HPF did respond to this bacterial challenge with viable *S. aureus* by secreting IL-8, which was found in equal concentrations in the apical supernatant as well as in the basolateral compartment. The equivalent levels of IL-8 in the apical and basolateral compartments of HPF cultures are not surprising considering that fibroblasts do not form a barrier as tight as epithelial cells do. HPF constitute a weak physical barrier (Myerburg *et al*., 2007) and they do not show an increase in TER over time (see chapter 5). The electrical resistance is very low (around 10 Ω x cm^2) and just higher than background readings and could therefore be used as an indicator of viable cells on the TW. This low resistance suggests that chemokines like IL-8 and other small molecules can diffuse from the “apical” side to the “basolateral” side or vice versa along a concentration gradient. IL-8 was only released when *S. aureus* was applied to the apical side of HPF cultures or to both sides at the same time. The simultaneous challenge from both sides caused a stronger response in terms of IL-8 compared to the apical challenge. However, no IL-8 response was seen after the basal challenge of these mono-cultures. The increase in IL-8 response seen when cells were challenged from both sides may reflect a response to higher numbers of bacteria, or a response to direct interaction with bacteria from the apical surface, but has yet to be determined.

This different behaviour of HPF on collagen IV coated TWs compared to these cells grown on collagen IV coated 24-well plates is surprising and suggests that pro-inflammatory responses of HPF, at least for IL-8 secretion, were altered by the different growth support and by the establishment of a multicell-layer (Chapter 5) rather than a mono-layer as it is seen on plastic ware (Chapter 3). Both the TWs and the 24-well plates were collagen IV coated and this should therefore not play a role in the different response observed. A significant difference is that cells cultured on TWs are supported on a semi-permeable membrane, which appears to support the formation of a multi-layered cell structure, even in these non-polarised fibroblasts. The "basal" cells which are nearest to the membrane do not have much freely exposed membrane surface area for the apically-applied *S. aureus* to adhere to, whereas the upwards facing cell surface membranes are completely accessible for the bacteria to adhere to. Whether HPF can show a certain degree of polarity (e.g. receptor expression) when grown on TWs and therefore whether these responses to apical bacteria were due to the freely exposed surface area with different receptors for bacterial adhesins cannot be answered yet. As already mentioned and as shown in figure 4.22, HPF grew into a multicell layer when cultured on TWs, whereas cells grown on plastic ware coated with collagen IV form a mono-layer (routine light microscopy observation of cells; Chapter 3), however whether this multiple cell layer formation is responsible for the "polarised" response of fibroblasts remains to be determined.

The epithelial cell behaviour was also different comparing submerged mono-cultures and mono-cultures at ALI. Under submerged conditions only Calu-3 showed an increase in IL-8 release after exposure to viable *S. aureus*, whereas C38 and IB3-1 did not respond at all and under these conditions *S. aureus* did not have any effect on epithelial cell viability. This different inflammatory response to *S. aureus* by these cell lines suggests that *S. aureus* or secreted virulence factors are able to bind to, and therefore stimulate, Calu-3 but not to the other two cell lines. Which receptor exactly was involved in the Calu-3 pro-inflammatory response is unknown, it is established that asialoGM1 can serve as a receptor for *S. aureus* (Ratner *et al*., 2001), but this receptor has yet to be demonstrated on Calu-3 cells. It was unexpected to see no pro-inflammatory responses from C38 or IB3-1 considering that these cell lines have been shown to express asialoGM1, even under submerged conditions (DiMango *et al*., 1995) and to elicit an IL-8 response, asialoGM1 and TLR-2 need to be co-mobilized into a lipid raft (Soong *et al*., 2004), and it has been demonstrated previously that C38 and IB3-1 two cell lines express functional TLR-2 and that permits cellular response to *P. aeruginosa* by activating NF-κB (Firoved *et al*., 2004). Calu-3 have also been shown to express TLR-2 at the level of mRNA and protein (O'Grady,

2007), but surface expression has not been confirmed. Such studies would provide a useful insight into the reasons behind the difference in response of these epithelial cell lines.

The cellular responses were different when epithelial cell lines were cultured on TWs at ALI. Here, C38 and IB3-1 showed a significant decrease in cell viability following all three challenges with viable *S. aureus*. This shows that, when grown at ALI, these two cell lines appear more susceptible to bacterial infection than under submerged conditions, suggesting that cell morphology and differentiation plays a role possibly in terms of receptor expression on the apical and basolateral surface membranes. These data also confirm the importance of producing physiologically relevant models- the differentiated epithelial cells that are produced following ALI (figure 4.11 and 4.12) closely resemble the native human airways and therefore these responses to infection are assumed to more closely resemble those seen *in vivo*.

Another important result to note was the difference between the models of normal and CF airways. TER was only affected in IB3-1 cells and only following the simultaneous challenge from both sides, which indicates a higher susceptibility of these cells (and thus the CF model) to viable *S. aureus*. Surprisingly even though there is significant reduction in cell viability, no increase in IL-8 secretion was observed at all. This decrease in viability in the apparent absence of inflammation is quite interesting considering that viable *S. aureus* will secrete several virulence factors, which usually induce an inflammatory response. It has been shown though that on one hand *S. aureus* is able to induce apoptosis and on the other hand that epithelial cells are able to ingest apoptotic cells themselves through efferocytosis. Even though this process is slower compared to professional phagocytes clearing apoptotic cells, these results could indicate that epithelial clearance of apoptotic cells is rapid enough to prevent an inflammatory response. Effective apoptotic cell clearance, before the damaged cell release internal cell compartments and noxious contents, is known to actively suppress proinflammatory responses (Vandivier *et al*., 2006). The decrease of cell viability seen here is therefore suggested to happen through apoptosis without inducing a pro-inflammatory IL-8 response.

In contradiction to this hypothesis, it has been reported for primary CF epithelial cells, as well as for IB3-1, that CFTR negative cells are not able to clear apoptotic cells, which will then accumulate and induce IL-8 release by CF cells (Vandivier *et al*., 2009). However, in support of these suggestions made here it has been shown that after 24 h infection with *S. aureus* a CF (CFT-1) and a non-CF (LCFSN) epithelial cell line became apoptotic upon internalising the bacteria. No difference in internalisation was seen between CF and non-CF cells (Kahl *et al*.,

2000). Additionally *S. aureus* is also able to kill epithelial cells, when a certain threshold of secreted autoinducer by *S. aureus* is reached, quorum sensing activates production of *S. aureus*'s virulence factor α-toxin, which has been shown to bind to a receptor called ADAM 10 and as a pore forming protein, α-toxin is able to lyse cells. At low level concentrations of α-toxin mainly apoptosis is induced (Essmann *et al*., 2003). To fully elucidate the decrease in cell viability seen here, further investigations will be needed.

In contrast to the other two cell lines the cell viability of Calu-3 was only affected by the simultaneous challenge with *S. aureus* from both sides, indicating that these cells are less susceptible to viable *S. aureus*. After the three challenges no IL-8 increase was observed not even in the presence of decreased cell viability, which was already discussed above. For Calu-3, the TER was significantly lower following the apical and apical/basolateral challenge, which suggests that apical exposure is necessary to perturb cell layer integrity. It has been shown before that *S. aureus* is able to disrupt tight junctions of 16HBE polarized cell layers in a 24 h period and the bacterial traversed the cell layer and were recovered from the basal compartment (Soong *et al*., 2011).Basolateral bacteria were not investigated in the current study, but again it is surprising that *S. aureus* did not evoke a pro-inflammatory response and this therefore needs further investigation.

Altogether these results show that there is no direct correlation between a decrease in TER and decrease in cell viability. Neither could the IL-8 secretion be directly linked not be directly linked to decreased cell viability and further studies will be elucidating whether these phenomenon is down to an anti-inflammatory effect of apoptotic cell clearance.

These findings were also observed for the co-cultures consisting of HPF together with one of the epithelial cell lines. The cell viabilities of HPF-C38, HPF-IB3-1 and HPF-Calu-3 were affected in two cases each. First it was shown that all three co-cultures showed a decrease in cell viability after the apical/basolateral challenge and secondly it was observed that the cell viability of HPF-C38 was decreased following the basal exposure to viable *S. aureus*, whereas HPF-IB3-1 and HPF-Calu-3 showed a decrease in cell viability after the apical challenge. This decrease following the apical challenge was not observed in Calu-3 mono-cultures, where following the apical challenge no change in cell viability was observed. The fact that HPF-IB3-1 and HPF-Calu-3 show a similar pattern regarding the cell viability suggests modulation of cell survival possibly based on the addition of subepithelial fibroblasts is not dependant on the expression of CFTR.

Observations of TER following challenge with viable *S. aureus* reveal that in all co-culture models the TER is significantly decreased after all three different ways of exposures to *S. aureus*. Even though the TER values of epithelial mono-cultures are comparable to the one established in the co-cultures models, it seems that the epithelial cell layer integrity of these cells in co-culture is more readily compromised than in mono-culture.

The induced proinflammatory responses of co-cultures were also found to be different compared to the mono-cultures, which is plausible as fibroblasts in mono-culture respond to *S. aureus* challenges. Interestingly in mono-culture HPF responded to apical challenges, where in the co-cultures they are subepithelial and the upwards facing surface membrane is not accessible for direct contact of *S. aureus* with HPF. This interaction can only occur from the basal side. However a basal response (potentially of fibroblast origin) was only registered for HPF-IB3-1 after basal exposure. HPF-IB3-1 did not show a significant increase of IL-8 following the simultaneous apical/basal challenge but this might be down to the huge decrease in cell viability seen here. HPF-C38 and HPF-Calu-3 only responded to the simultaneous apical/basal challenge but not anymore to the apical challenge indicating a supporting role of the fibroblasts for the epithelial cell layer. As mentioned before it has been shown that fibroblast support epithelial cell differentiation and also ciliogenesis (Myerburg *et al*., 2007). Interestingly, it seems the subepithelial HPF cannot exhibit this support for IB3-1.This might indicate that there is a difference in direct cell communication- in terms of either the signalling molecules or the necessary receptors- for IB3-1 and HPF compared to the other two models. Whether this is down to IB3-1 expressing mutated CFTR, or a knock-on effect of genes modified by mutated CFTR needs to be further investigated.

It is important to note that for HPF-C38 and HPF-IB3-1, baseline IL-8 secretion is much higher than in mono-cultures. Why there is an increase in baseline IL-8 release in these co-cultures has not yet been explored, but it appears that there is some co-stimulation of epithelial responses by the presence of proliferating fibroblasts, and vice versa. Understanding this mechanism of communication is one important aim of establishing these models.

No pattern of IL-8 secretion could be identified comparing all three co-cultures, which is difficult as it is impossible to tell which cell type did secrete the IL-8, how much was secreted by each of the cell types or in which direction it was secreted by the individual cell populations. This is similar for cell viability, it is impossible to tell whether decrease in cell viability is down to only one cell type or down to both cell types being affected.

These results show that the addition of fibroblasts modulates epithelial cell behaviour in response to *S. aureus*. How exactly intercellular communication, between fibroblasts and epithelial cells influences the three measured parameters is not known but the challenge of understanding these mechanisms of communication needs to be taken up and may lead to novel ideas for targeted therapies. Co-cultures of fibroblasts and epithelial cells should help to move forward in the right direction to investigate molecular mechanisms, whereas animal models here are complicated by too many variables, which lead to lack of properly controlled experimental conditions (Skibinski *et al*., 2007).

7.5.2 *B. cepacia* challenge of submerged mono-cultures and mono- and co-cultures at ALI

The second CF relevant organism used throughout this work was *B. cepacia*. This bacterium has become a problem throughout the last 2 decades as a pathogen in patients of cystic fibrosis (LiPuma, 2003). Furthermore *B. cepacia* infections in CF patients have been correlated to a rapid decline of lung function as well as septicaemia (Sajjan *et al*., 2001), which differentiates this CF pathogens from the others discussed here.

For mono-cultures under submerged conditions it was observed that viable *B. cepacia* did not have an impact on cell viability of the employed cells. However, for IB3-1 and Calu-3 submerged mono-cultures an increase of IL-8 was observed following the 24 h exposure to viable *B. cepacia*. Calu-3 have been shown before to respond to *B. cepacia* exposure under these conditions. In the same study A549, 16HBE14o- and CFBE41o- were analysed for their ability to respond to *B. cepacia*. Calu-3 were found to secrete the highest level of IL-8 compared to the other cell lines, similar to what was found here (Kaza *et al*., 2011).

Interestingly in response to this bacterium, IB3-1 secretes much more IL-8 compared to C38. At baseline IB3-1 secreted 10 times more IL-8 than C38 and after the challenge this increased to almost 300 times higher IL-8 in IB3-1 supernatant compared to C38. This higher baseline IL-8 also correlates with other studies. Kaza *et al*. (2011) found that the CF cell line CFBE41o- secreted more IL-8 when unstimulated compared to 16HBE14o- and it has also been reported before that 16HBE14o- secrete less IL-8 compared to IB3-1 (Schwiebert *et al*., 1999, Kaza *et al*., 2011).However, after stimulation with different *B. cepacia* strains, Kaza *et al*. (2011) reported CFBE41o- secreted the lowest level of IL-8 compared to the other cell lines used, which is in contrast to our findings (Kaza *et al*., 2011). A report by Fink *et al*. (2003) pointed out that in response to several different *B. cepacia* strains IL-8 responses of IB3-1 and C38 were only slightly different (Fink *et al*., 2003). However, for studies using *P. aeruginosa* as stimulus it was reported

that IB3-1 responds with constitutively higher secretion of IL-8 when compared to C38 (DiMango *et al*., 1998). These different studies show that there is variability in the concentrations of IL-8 released after 24 h exposures. There are several points to remember when looking at these variable data. Cell lines that do not originate from the same patient and might have different CFTR mutations are difficult to compare to each other. Furthermore laboratory differences in handling and differences of growth conditions do play a role. For respiratory epithelial cells it is also important to remember that cell lines originate from different parts of the airways and therefore react differently to different stimuli. Whereas Calu-3 represent a serous type cell (Shen *et al*., 1994), A549 are a type II epithelial cell line (Lieber *et al*., 1976) and C38 (Flotte *et al*., 1993) and IB3-1 (Flotte *et al*., 1992) are bronchial cells.

For HPF mono-cultures grown under submerged conditions on TWs the cell viability is not affected by exposure to live *B. cepacia*, similar to what was seen in submerged cultures. However, the pro-inflammatory response was again different when grown on TWs. These mono-cultures did respond following the exposure to viable *B. cepacia* with a tremendous increase of IL-8 in both compartments after all three challenges of the model system. Interestingly the IL-8 concentration after the basal and the apical/basal concentration was higher in the basolateral medium than in the apical supernatant suggesting that upon basal stimulation with this bacterium, IL-8 is secreted preferably to this compartment as the concentration was not equilibrated across this weak barrier after 24 h this seems to also be a continuous release. Fibroblasts of the airways have not been extensively studied in connection with CF but the strong responses of HPF found in terms of IL-8 secretion indicate that HPF play an important part in proinflammatory responses to bacterial infections, especially when the bacterium, such as *B. cepacia* is known for its ability to disrupt TJs and cause septicemia (Sajjan *et al*., 2001). This enables the organism to traverse the epithelial cell layer and get into direct contact with the subepithelial fibroblasts. This was already observed in CF patients, where *B. cepacia* was found in the terminal and respiratory bronchioles- much further down the respiratory tract than other pathogens. In addition intercellular migration was seen as well, again supporting that this bacterium crosses the epithelial barrier (Sajjan *et al*., 2001). It has also been shown for polarized 16HBE, for example that after only 4 h after infection, *B. cepacia* was recovered from the basolateral medium of this culture (Kim *et al*., 2005), which supports the idea that *B. cepacia* would be able to reach the subepithelial fibroblasts and directly induce a proinflammatory response, which might play a huge role in CF airways, considering that HPF release between 1500 – 4000 pg/ml here depending on the challenge and compartment analysed.

Interestingly the polarized epithelial cell mono-cultures presented here did not show a decrease in TER, even though Kim *et al*. (2005) has shown that TJ can be disrupted in a much shorter time frame than 24 h (Kim *et al*., 2005). Only for Calu-3 a decrease in TER was observed after the simultaneous apical/basal challenge for 24 h with live *B. cepacia* similar to what was shown for 16HBE. Different *B. cepacia* strains were used by Kim *et al*. (2005) and all strains display different abilities in crossing well polarized 16HBE epithelial cells (Kim *et al*., 2005). Furthermore the cell viability of these epithelial mono-cultures was not compromised by these exposures to viable *B. cepacia*. This can be explained on one hand by the idea that traversing the epithelial cell layer does not cause cell injury or cell death (Kim *et al*., 2005) and on the other hand by *B. cepacia*'s ability to survive intracellularly in epithelial cells suggesting that this bacterium has an intracellular phase during pulmonary infections of CF patients (Martin and Mohr, 2000).
The only significant changes were observed in IL-8 secretion, which was increased in all three cell lines after different challenges for 24 h exposure to live *B. cepacia*. *B. cepacia* J2315 is known to be one of the most potent inducers of inflammatory responses, transmissibility and fatal infections (Fang *et al*., 2011, Drevinek *et al*., 2008, Martin and Mohr, 2000) and strong proinflammatory responses to the intact bacterium were expected here.

In comparison to C38, IB3-1 secreted more than 3-fold more IL-8 to the apical compartment after apical challenges, including the simultaneous challenge. IB3-1 cells do not always show higher IL-8 at baseline compared to C38 but the magnitude of stimulated IL-8 secretion is much higher, similar to what was found here in submerged mono-cultures and found elsewhere before in response to *P. aeruginosa* (DiMango *et al*., 1998). There are some other interesting differences to point out though. C38 also responds to the basolateral challenge and interestingly C38 samples of basolateral medium showed a significant increase in IL-8, which was not seen IB3-1. Calu-3 responded similarly to IB3-1 after the apical challenges with significant higher IL-8 in apical supernatants but basolateral IL-8 was only found increased after the apical challenge. For all three cell lines though, the apical exposure to *B. cepacia* was definitely more immunomodulatory compared to basolateral exposures. For all three epithelial cell lines this pattern of IL-8 in apical supernatants was similar and interestingly there seems to be no additive response when challenged from both sides simultaneously as the final IL-8 concentration of this challenge is similar to the one found after apical exposure. The basolateral IL-8 measured was only significantly increased in C38 samples (all three challenges) and in the Calu-3 sample after apical challenge alone. These findings indicate that IB3-1 is unable to respond to the basolateral challenge and might suggest a basolateral receptor difference between CF and non-CF cells.

In vivo the epithelium is polarized with distinct apical and basolateral protein and lipid expression. *B. cepacia* can adhere to epithelial cells via both protein and glycolipid receptors, which could be presented differently in apical compared to basal membranes of these epithelial cells presented. The *B. cepacia* strain used here is a cable-pilus expressing one, which enables this bacterium to bind to secretory mucins as well as to CK13, for example. The stronger IL-8 response of IB3-1 compared to C38 could be related to the increased surface exposure of CK13 seen in chronic inflamed CF lungs (Sajjan *et al*., 2000, McClean and Callaghan, 2009). In addition to this, the apically expressed glycolipid receptor asialoGM1 (de Bentzmann *et al*., 1996a) has been shown to bind *B. cepacia* and other bacteria, such as *P. aeruginosa* and *S. aureus* (Krivan *et al*., 1988, Firoved *et al*., 2004). In relation to CF this receptor plays a significant role as it displays an apical binding site for this bacterium but also because superficial asialoGM1 has been found to be more numerous on primary CF cells (Saiman and Prince, 1993). This has also been shown for IB3-1 cells compared to C38 (DiMango *et al*., 1998), which would explain the stronger inflammatory response seen in IB3-1 apical supernatants compared to C38.

The principles of *B. cepacia* binding and inducing a proinflammatory response to airway epithelial cells are the same in co-cultures. However, the basolateral membrane of epithelial cells is not directly accessible as in co-cultures the fibroblasts are located subepithelial. Instead the fibroblast membrane is accessible after bacteria managed to move through the semipermeable membrane and the bacteria can then travers across the HPF layer and reach the epithelial cells to induce an proinflammatory response. A striking difference is that after the simultaneous apical/basal challenge the cell viability was decreased in all three co-culture models. For HPF-IB3-1 additional decrease in cell viability was seen after the apical as well as after the basal challenge with viable *B. cepacia*, pointing out that this co-culture is most susceptible out of these three co-cultures.

B. cepacia produces a haemolytic toxin that is able to induce apoptosis in cultured macrophages (Martin and Mohr, 2000) but whether this virulence factor plays a role in epithelial apoptosis is unclear. Furthermore another group has recently shown that *B. cepacia* is also able to induce apoptosis in airway epithelial cells (the BEAS 2B bronchial epithelial cell line) only 3h after infection (Moura *et al*., 2008) but the difference between CF and non-CF has not been addressed and so the role and the cause of apoptosis in the pathogenesis of *B. cepacia* is not completely understood and remains under investigation.

The TER however was not decreased in HPF-C38 and HPF-IB3-1 but it was decreased in all three cases of challenges in HPF-Calu-3 co-cultures. As already discussed earlier *B. cepacia* is known to be able to disrupt tight junctions but why there is such a difference between HPF-Calu-3 compared to HPF-C38 and HPF-IB3-1 is not known. The difference might be because of different adhesion mechanism used by *B. cepacia* for these different cell lines depending on availability of receptors.

IL-8 secretion was found to be in a similar pattern compared to mono-cultures in case of HPF-C38 and HPF-IB3-1 with the difference that HPF-IB3-1 showed an apical increase in IL-8 after the basal challenge and in correlation with data of mono-cultures IB3-1 co-culture still produces more IL-8 compared to HPF-C38. HPF-Calu-3 only showed a significant increase in IL-8 secretion after the simultaneous challenge from both compartments but not following the apical challenge. These results indicate that HPF modulate epithelial cell behaviour at ALI and points out that they did not inhibit epithelial cell function. Assuming that the apical increase in IL-8 following the basal challenge is partially due to the subepithelial fibroblasts this is an interesting finding and needs to be investigated further. One receptor that could induce IL-8 secretion by fibroblasts upon *B. cepacia* binding is TNFR1, which is known to be present in fibroblasts and epithelial cells (Saldias and Valvano, 2009) and has been shown to serve as binding site for *B. cepacia*. Interestingly this receptor has also been found to be expressed more numerous on primary CF cells compared to non-CF cells (Sajjan *et al*., 2008), which supports the stronger inflammatory response seen by HPF-IB3-1 co-cultures as well as the stronger response of IB3-1 mono-cultures when these are compared to the corresponding C38 mono- or co-culture.

7.5.3 *P.aeruginosa* challenge of submerged mono-cultures and mono- and co-cultures at ALI

As already mentioned before *P. aeruginosa* is the most common bacterial infection found among CF patients and it is one of the main research goals in CF to elucidate how the reduction, elimination or dysfunction of CFTR leads to this hypersusceptibility of CF patients to this bacterium. The *P. aeruginosa* strain used here was isolated from a CF patient but no further patient details are available in terms of age, level of lung disease and treatments, for example.

P. aeruginosa is known to produce and secrete several virulence factors, including elastase (Azghani *et al*., 1992), exoenzyme S (Epelman *et al*., 2000), exotoxin A (Azghani, 1996) and pyocyanin (Denning *et al*., 1998) for example, which support the persistence of *P. aeruginosa* infections in CF patients. Virulence factors definitely contribute to the pathogenesis and

establishment of infection of these bacteria and understanding the molecular and cellular events that allow adherence of *P. aeruginosa* and the establishment of chronic infection is vital to elucidate CF lung pathogenesis. The established mono- and co-cultures were exposed to live *P. aeruginosa* to analyse the affect of this bacterium on cell viability, TER and IL-8 secretion.

Under submerged conditions in 24 well plates, HPF mono-cultures showed a significant decrease in cell viability (decreased by 1.9-fold) as well as a significant, almost forty-fold increase in the release of the inflammatory cytokine IL-8 after exposure to viable *P. aeruginosa*. The ability of *P. aeruginosa* to adhere to human lung fibroblasts was shown before and it was highlighted that bacterial elastase, which degrades surface bound and ECM fibronectin, leads to increased adherence of this bacterium, for example (Azghani *et al*., 1992). Bacterial elastase has also been shown to cause increase of epithelial cell layer permeability (rat alveolar epithelial cells) (Azghani, 1996), which indicates that *P. aeruginosa* is able to disrupt the protective barrier and possibly reach the subepithelial fibroblasts, where the bacterium can then induce additional IL-8 responses, which could be part of the extensive IL-8 concentrations found in CF. Furthermore it has been shown that normal human lung fibroblasts were stimulated to secrete IL-8 (analysed on mRNA and protein level) in response to the exposure to one of *P. aeruginosa*'s autoinducers, which is freely diffusible and part of the quorum sensing system of this organism to regulate gene expression. As this small molecule has been shown to not only be present directly around the bacteria but also in the underlying epithelial cells it was suggested that this virulence factor can also reach the underlying fibroblasts to induce additional pro-inflammatory response. In that study the fibroblastic response was also found to be longer lasting than the one of epithelial cells, highlighting the importance of this cell type in inflammation (Smith *et al*., 2001). These two reports show that there is a need to elucidate the importance of fibroblasts during lung infection in CF and the role they play, which possibly helps to understand CF lung pathogenesis further.

In submerged cultures of C38, IB3-1 and Calu-3 a strong induction of IL-8 release was observed in response to live *P. aeruginosa*. Following this challenge IL-8 concentrations in IB3-1 supernatants were found again to be about 30 times higher when compared to C38, which is the corrected counterpart of IB3-1 that expresses functional CFTR. Similar results were found for primary submerged CF and non-CF epithelial cells by Joseph *et al*. (2005), when stimulating primary CF and non-CF epithelial cells with *P. aeruginosa* PAO1. They demonstrated that a constitutive and increased NF-κB activation was associated with increased IL-8 mRNA after exposure to PAO1 in CF cells. This group also analysed cell viability before and after exposure and found a decrease in cell viability by almost 60%, with no differences seen between CF and non-CF (Joseph *et al*.,

2005), whereas in the present study, cell viability of C38 and IB3-1 was stable after24 h exposure to *P. aeruginosa* under submerged conditions. This different observation of cell viability could be due to differences between transformed cell lines and primary cells or could also be down to the *P. aeruginosa* strain used. In another study PAO1 was used at 10^7 CFU/ml to stimulate submerged cells and again CF cells were found to secrete more IL-8 (4-fold) in response compared to non-CF cells, which was proposed to be down to asialoGM1 expression on CF cells (DiMango *et al*., 1995). These findings are consistent with the results found here in terms of IL-8 secretions.

For Calu-3 the third epithelial cell line used in these experiments a much stronger response to *P. aeruginosa* was seen compared to C38 and IB3-1 but these adenocarcinoma-derived cells have a baseline secretion of IL-8 higher than the *P. aeruginosa* induced response of IB3-1. As already mentioned above, this cell line also showed an increased IL-8 secretion after the exposure to *S. aureus* and *B. cepacia*. The cell viability, however, was not affected here either, similar to what was seen for C38 and IB3-1.

When these cell lines were cultured on TWs the effect of *P. aeruginosa* on the cells was different. HPF were, as for the other bacterial challenges, cultured under submerged conditions on TWs, which enabled challenge of the cells from the apical side, basolateral side or from both sides at the same time. A decrease in cell viability as it was seen in submerged cultures could not be observed for these challenges carried out on TWs. Again, as already discussed above, this indicates a difference between submerged mono-cultures grown on plastic and submerged mono-cultures grown on TWs, whether the differences are down to the now established multi-cell layer remains to be investigated but it has been shown before that fibroblasts that were dispersed in collagen gels represented a mesenchyme equivalent for epithelial cell differentiation (Tsai *et al*., 1994), which represents the *in vivo* situation closer than a mono-layer of fibroblasts and is suggested here to play a role in infection and inflammation responses.

Exposures to viable *P. aeruginosa* from the apical side and from the apical/basal side simultaneously caused a massive increase in IL-8 concentration in apical supernatants of this mono-culture in response to *P. aeruginosa*. Only after the simultaneous challenge from both sides at the same time there was a significant increase of IL-8 in the analysed basolateral medium. Interestingly, in contrast to the mostly equilibrated basal and apical IL-8 concentrations found after *S. aureus* and *B. cepacia* stimulation, after this challenge there was more IL-8 found in apical secretions without equilibration after 24 h. This suggests that there may be defined

responses to specific pathogens by the HPF and that these interactions might partially cause a directional secretion of IL-8.

For the epithelial cell mono-cultures of C38 and IB3-1 at ALI, the cell viability following exposure to live *P. aeruginosa* was significantly reduced after all three challenges irrespective of the site of exposure. This decrease in cell viability was not observed for Calu-3 mono-cultures after the 24 h challenge and highlights cell line differences of these epithelial cells. The cell layer integrity was analysed before and after the challenges and found to be intact as no changes in TER were observed for C38 and IB3-1 comparing before and after, whereas the cell layer integrity of Calu-3 was significantly lower after all three challenges with live *P. aeruginosa*. In mono-cultures of C38 and IB3-1 there was a significant increase of apical IL-8 concentration after apical exposures to *P. aeruginosa*, including the simultaneous challenge from both sides at the same time. Similar to what was observed before, IB3-1 produces about three times as much IL-8 in response to live *P. aeruginosa* compared to C38. Another interesting difference to note is that after the simultaneous challenge from both sides at the same time an increase in basolateral IL-8 concentration was observed for C38 mono-cultures but not for IB3-1. The pro-inflammatory response found for Calu-3 mono-cultures was quite different compared to IB3-1 and C38. IL-8 concentrations were significantly increased after basal and after the simultaneous challenge from both sides but not following the apical exposure. Again it is important to keep in mind that these are different cell lines with different origins.

P. aeruginosa has been reported to modulate lung epithelial cell functions as well as having a direct cytotoxic affect on these, which has been shown *in vivo* and *in vitro* (Lau *et al*., 2005, Rajan *et al*., 2000). One of *P. aeruginosa*'s virulence factors that is able to induce apoptotic and necrotic cell death is exotoxin A, which is has been shown to bind to the basolaterally presented receptor 2-macroglobulin, which supports internalisation of this virulence factor and is only expressed in polarized epithelial cells (Kounnas *et al*., 1992) therefore this could explain why the decrease in cell viability was not observed in submerged cultures. However it is interesting that no decrease in cell viability was observed for Calu-3 mono-cultures at least in this respect following the basal challenge and it is surprising that no IL-8 response was seen in C38 and IB3-1 after the basal challenge, even though there is a decrease in cell viability. This was already discussed above as similar results were seen for *S. aureus* in terms of cell death in conjunction with no IL-8 increase. If the decrease in cell viability is down to apoptosis a pro-inflammatory response would not be expected (Vandivier *et al*., 2006). Apical challenges of C38 and IB3-1 showed a decrease in cell viability and a strong IL-8 response, whereas in Calu-3 there is no

decrease in cell viability and no response to the apical challenge. This difference in response and direct affect of viable *P. aeruginosa* could be down to several reasons. One plausible explanation is the difference in the antibacterial activity of apical secretions. For Calu-3 it has been shown that the apical surface fluid exhibits antibacterial activity against *P. aeruginosa*. Whether this is true for C38 and IB3-1 grown under these conditions is unknown. However cell viability of C38 and IB3-1 is also decreased after the basal exposure. Controversially Calu-3 showed a significant induction of IL-8 after basal exposure without cell viability decrease, which was not seen in the other two cell lines. Whether there is an expression difference of macroglobulin-2-receptor and possibly other receptors between these two cell lines and Calu-3 is not known and it remains to be elucidated why there are such different epithelial cell responses following the exposure to P.aeruginosa.

C38 and IB3-1 responded to apical challenges with apical IL-8 release and as already mentioned before the apically expressed asialoGM1 (de Bentzmann *et al*., 1996a) receptor is a potential candidate to initiate this pro-inflammatory response. AsialoGM1 has been shown to be much more numerous in CF cells and that *P. aeruginosa* pili bind to this receptor (DiMango *et al*., 1995) as well as flagella, which both serve as adhesins and induce IL-8 secretion in epithelial cells. The non-mucoid strain used in these experiments was originally isolated from a CF patient and even though it often was reported that irreversible genotypic modulation from a non-mucoid to a mucoid strain occur in the CF lung environment this was not the fact here (Martin *et al*., 1993). Also it has been shown that this is usually accompanied by another genotypic modulation that has been documented for *P. aeruginosa* strains in CF lungs. This is transformation from a motile to a non-motile bacterium, which does not express flagella anymore (de Bentzmann *et al*., 1996a, Mahenthiralingam *et al*., 1994). It has been suggested that this transformation also affects pili (Pier *et al*., 1992). If this genotypic change to a non-motile phenotype occurred then this would exclude asialoGM1 to be responsible for the increased IL-8 secretion in IB3-1 mono-cultures and it has been shown for several clinical isolates of *P. aeruginosa* that they do not show adhesion to asialoGM1 (Schroeder *et al*., 2001b).
Another possible receptor that has been shown to be a binding site for *P. aeruginosa* is CFTR itself (Pier *et al*., 1996), which bind the bacterium and it is then internalized to clear the infection by shedding of epithelial cells. Epithelial cells start shedding into the ASL to clear the infection and it was also found that non-CF cells internalised *P. aeruginosa* at a very poor rate (Pier *et al*., 1996). Assuming that CFTR plays a role here, the C38 data fit quite well to this story as there is a moderate IL-8 response, which will be initiated upon binding and possible cell-cell communications and this is accompanied by a decrease in cell viability, which is down to the

increase in cell shedding as an attempt to clear the infection. Looking at IB3-1 there is a similar pattern of pro-inflammatory response but three times as high compared to C38 and also there is a similar decrease in cell viability, which would not be expected if this is down to internalisation of *P. aeruginosa* upon CFTR binding, which should not be present in the apical surface membrane of IB3-1. And again it does not explain the decrease in cell viability following the basal exposure to viable *P. aeruginosa*. Calu-3 mono-cultures only responded to the basal and the simultaneous challenge with live *P. aeruginosa*, even though they have been shown to express CFTR (Shen *et al*., 1994). Additionally it has been suggested that mucoid clinical isolates loose the binding site for CFTR (Schroeder *et al*., 2001a) and so that may not be the cause of IL-8 secretion here at all.

In another study it was shown that *P. aeruginosa* binds more readily to basolateral membranes of polarized epithelial cells to induce an inflammatory response (Pier, 2002), like it was seen here for Calu-3. This means that *in vivo* the electrical resistance and therefore cell layer integrity is lowered by the massive influx of PMNs, which then creates access for the bacteria to the basolateral side of the epithelium to which bacteria can adhere and can be internalised (Goldberg and Pier, 2000).

To confirm what exactly causes the differences in IL-8 response among epithelial cells but also between CF and non-CF cells (magnitude) further analysis is required. When considering that *P. aeruginosa* exhibits several different virulence factors, which are regulated by different autoinducers and quorum sensing systems, it is impossible here to appoint one or two factors that cause these responses. There is a report that concentrated on the altered gene expression of *P. aeruginosa* grown in different sputum samples for example and the list of genes that were altered in expression is enormous (Palmer *et al*., 2005) but by developing models like these and also the co-culture models it will be possible to dissect different responses and different stimuli.

For the co-cultures of these three epithelial cell lines the following results were observed. For HPF-C38 and HPF-IB3-1 cell viability was decreased significantly after the simultaneous challenge with *P. aeruginosa* from the apical and basal side at the same time but for HPF-IB3-1 cell viability was decreased by almost 70% compared to only 33 % decrease of cell viability seen for HPF-C38 again pointing out the higher susceptibility of CF co-culture system. Then it was interesting to find that the cell viability of HPF-C38 was decreased after the basal challenge with viable *P. aeruginosa* but here the cell viability for HPF-IB3-1 was unaffected but for the apical challenge this was observed to be the other way around. As already discussed for mono-cultures of IB3-1

and C38 it is difficult to identify the exact factor that causes this susceptibility, especially in a more complex model with a subepithelial HPF cell layer. Almost nothing has been reported about pulmonary fibroblasts and their role in these kind of inflammatory responses and availability of receptors on these fibroblasts, for example. For HPF-IB3-1 a decrease in TER was found after all three challenges, which was not observed in HPF-C38, where the TER was found to be stable, which was also found to be stable of HPF-Calu-3, which is a contrast to the decreased electrical resistance found in mono-cultures of Calu-3. AS already mentioned for mono-cultures, bacterial elastase has been shown to increase cell layer permeability, for example (Azghani, 1996) and the decreases seen here could be explained by active elastase in the cell supernatant.

For HPF-C38 there was only a pro-inflammatory response following the basal and simultaneous apical/basal challenge with viable *P. aeruginosa*, which are the challenges after which also the cell viability was decreased. It seems that the addition of fibroblasts modulates IL-8 release of epithelial cells and possibly also the other way around. The IL-8 release of HPF-IB3-1 is also different to what was observed in IB3-1 mono-cultures, as there is now a strong response to the basal challenge but not after the simultaneous apical/basal challenge. The huge decrease of cell viability following the apical/basal challenge could be the reason that almost no increase of IL-8 was seen though.
HPF-Calu-3 showed a different pro-inflammatory response pattern as well, when compared to the mono-cultures. The co-culture still showed a significant increase after the apical/basal challenge but not anymore following the basal challenge. Furthermore did the co-culture respond to the apical challenge, which was not observed for the mono-culture.

As single virulence factors of *P. aeruginosa* were not investigated here it is impossible to comment on direct influences of them on these co-cultures. One IL-8 stimulatory factor though that would be expected to be found in high density bacterial cultures is bacterial DNA, which has been shown to induce IL-8 secretion in CF cells like IB3-1 and CuFi1 cells and non-CF epithelial cells (NHBE) but IL-8 secretion was higher in CF cells, which is consistent with findings in this study (Delgado *et al*., 2006). However, it is difficult to analyse IL-8 data accurately because of the ongoing cell death in HPF-IB3-1 co-culture and therefore the direct comparison to HPF-C38 is not possible. Interesting is as well that in all three mono-cultures the IL-8 response to live *P. aeruginosa* is found mostly on the apical side of polarized cultures, whereas in the co-culture system basolateral IL-8 is observed as well after basal stimulation. The presence of fibroblasts seems to modulate the pro-inflammatory response in a way that we would expect *in vivo*, where

cytokines in the airway lumen prime the incoming neutrophils and keep them active, whereas the basolateral secreted IL-8 would build up a concentration gradient in the tissue to recruit more immune cells to the site of infection. It has been shown before that neutrophil migration across endothelial and epithelial cell layers is dose and time-dependant (Smart and Casale, 1993), which supports the idea that apically secreted IL-8 is important to pull the neutrophils all the way through the cell layer and equally important is basal IL-8 to get neutrophils crossing the endothelial cell layer *in vivo.* It is suggested that fibroblasts do play an important role in fulfilling the correct inflammatory response in healthy airways if necessary and it is suggested that fibroblasts could play an important part in CF lung pathogenesis.

Collectively these data show that HPF are able to respond to infection in terms of IL-8 secretion and would appear to be actively taking part in pro-inflammatory signalling. Fibroblasts play an important role in inflammatory responses as shown here and are not just structural support cells. Therefore are the established co-culture models presented here a valuable need for further investigation to shed some light on the input of these cells in healthy and in CF airways. IB3-1 mono-cultures do respond with significantly more IL-8 secretion compared to C38, which is also mainly the case in these co-cultures, indicating that HPF support epithelial cell behaviour to infection. In general *B. cepacia* is the most potent inducer of IL-8 secretion, which is also seen in mono- as well as in co-cultures. Similar results were found for the cell layer integrity. If it was affected by live bacterial exposure, TER decreased to a higher degree in IB3-1 or HPF-IB3-1 cultures. Again CF cells susceptibility to infection was also reflected by cell viability decreases, which were mainly higher in CF compared to non-CF.

Furthermore the difference of fibroblast responses when cultured on different supports (TWs or plastic, both collagen IV coated) needs further investigation. Fibroblasts are now recognised as active, participating cells, which play vital roles in inflammatory responses, rather than just being structural support cells, the co-cultures were established to gain more information about their inflammatory role in infection and will help to study epithelial cell and fibroblast interaction and communication.

8 Chapter 8 Inflammatory response to heat-inactivated (HIA) bacteria

8.1 Introduction

In this chapter the impact of heat-inactivated *S. aureus*, *B. cepacia* and *P. aeruginosa* on the cell viability, the TER of cultures at ALI and the pro-inflammatory response in terms of IL-8 was investigated. Purified LPS has already been shown to have limited impact on the established mono-and co-cultures, in terms of their inflammatory response (chapter 6), and further it was shown that live bacteria can have very different affects on these cultures, which was outlined in chapter 7.

Heat-inactivation of bacteria is a method commonly used for the destruction of pathogenic bacteria, especially in the food industry, for example. Here thermal processing is commonly used to produce a safe product and enhance the shelf life of food. Heat inactivation of bacteria leads to irreversible changes and denaturation of membranes, ribosomes and nucleic acids (Lee and Kaletunc, 2002). The method used here involves heating bacterial suspensions with moist heat, close to boiling, which inactivates bacterial peptidal exotoxins completely (Gao *et al*., 2006). Washing of HIA bacteria before the cell suspension is adjusted to a standard bacterial cell density eliminates all inactivated exotoxins produced by these bacteria and any other secreted virulence factors and bacterial by-products. Different virulence factors and how they might affect the established mono- and co-culture models were discussed in chapter 7 and will not be taken into account here. Bacterial cell wall components, such as LPS of gram-negative bacteria is known as heat-resistant endotoxin but has been reported to be affected in its endotoxin activity when boiled at 100°C. At this temperature the endotoxin activity is reduced after only 15min (Gao *et al*., 2006). Here we used water at 80°C to heat inactivate the bacteria inactivation/cell death was confirmed by plating out samples on agar plates before and after the challenges.

8.2 Aims

In this chapter submerged mono-cultures as well as mono-and co-cultures at ALI were exposed to heat-inactivated (HIA) *S. aureus*, *B. cepacia* and *P. aeruginosa* to investigate inflammatory responses to these heat-killed bacteria, when exotoxins are eliminated from the experiment and only heat stable cell wall components were present. In addition cell viability and cell layer integrity were monitored alongside IL-8 release.

In order to achieve these aims the cell viability after exposure to HIA bacteria was investigated for all cell culture models presented.

Furthermore monitoring of the cell layer integrity by measuring TER before and after the challenges was essential to estimate the affect of HIA bacteria on the barrier formation of mono- and co-cultures at ALI.

Finally it was necessary to determine the pro-inflammatory response (IL-8 secretion) of these mono- and co-cultures after exposure to HIA bacteria.

8.3 Methods

8.3.1 Heat inactivation of bacteria

Colonies of *P. aeruginosa 50DR, B.cenocepacia J2315* and *S. aureus* were picked from a freshly cultivated MHA plate and grown in 50 ml MHB at 37 °C overnight in a shaker at 150 rpm. On the next day cultures were split into equal parts (one half was kept for experimental treatment of cell cultures with live bacteria) and one half was put into a waterbath maintained at 80 °C for at least 1 h. To be sure bacteria were alive prior to heat-inactivation and dead post heat-inactivation; samples were taken before and after placing the bacterial suspension into the waterbath. Every 15 mins a sample was taken from the waterbath to determine at which time point the bacteria were all killed. After successful heat inactivation (1 hour) the heat-inactivated bacterial cultures were spun down at 3500 x g for 15 min and washed in 10 ml sterile PBS. This was repeated three times before the culture's optical density (OD) was measured at 470 nm. The OD was then adjusted to an OD_{470nm} of 1.0, which corresponds to a bacterial cell number of approximately 1 x 10^9 CFU/ml for *S. aureus, B. cepacia* and *P. aeruginosa.* These bacterial suspensions were then diluted 1:10 to make a stock solution of 1 x 10^8 CFU/ml. The final bacterial cell density used in the experiments under submerged conditions or at ALI was 1 x 10^7 CFU/ml.

8.3.2 Cell viability assay – Cell Titer Blue™ (Promega)

Cell Titer Blue (CTB) is an endpoint assay that provides a homogeneous, fluorometric method to monitor cell viability. Viable cells, which are metabolically active, can convert resazurin (blue with little intrinsic fluorescence activity) into its highly fluorescent product, called resorufin (pink). CTB was directly applied to fresh serum free cell medium after samples have been collected and the plate was then incubated for 2 h at 37 °C prior to analysis. The fluorescent intensity was measured on a standard multiwell fluorescent plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with a 560 nm excitation, and 590 nm emission wavelength.

8.3.3 Transepithelial electrical resistance (TER) measurements

TER of mono- and co-cultures on TWs was measured using an Epithelial Voltohmeter with STX2 chopstick electrodes (World Precision Instruments) to monitor confluence and barrier formation

of the epithelial cell layer. TER was measured on day 14 or just before exposure to live bacteria to verify epithelial cell confluence and intactness of epithelial cell layer and TJ formation.

TER values reported in this work take into account the area of the cell layer (area of TWs = 0.33cm^2) and are expressed as Ωcm^2. Triplicate measurements were taken for TW and the background resistance, which was typically between 100-120 Ωcm^2 (cell-free collagen IV coated TW) was subtracted from the average of a triplicate measurement.

TER was measured immediately before and straight after the 24 h incubation with live bacteria to analyse whether this cell layer integrity was affected and changed compared to the control TWs.

8.3.4 Bacterial growth conditions

P. aeruginosa 50DR and *B. cepacia* J2315 were grown in MHB at 37°C and placed in a shaker at 150 rpm over night or were plated out on MHA plates and incubated in a 37°C incubator. Bacteria were also stored on MicroBank beads (Pro-Lab Diagnostics, Ceshire, UK) at -70°C until required.

8.3.5 Treatment of submerged mono- cultures and mono- and co-cultures grown at ALI with HIA bacteria

Submerged mono-cultures were set up exactly the same way as described in 2.17. On the third day of culture ITS-medium was replaced with ITS-medium containing approximately 1 x 10^7 cfu/ml of HIA bacteria and cells were incubated for 24 h. The same procedure was followed to expose mono-and co-cultures grown at ALI to these HIA bacteria. After 14 days of culture at ALI mono- and co-cultures were either apically, basolaterally or simultaneously from both sides, exposed to HIA bacteria. Control TWs with mono-and co-cultures were handled the same way but medium without bacteria was used.

Samples were then collected from submerged mono-cultures as well as from TWs (apical side and from the basolateral side) and these were then analysed for IL-8 concentration separately using an ELISA kit as described in 8.3.6. CTB was applied to all cultures to analyse cell viability. After 2 h incubation with CTB the medium was removed and cell viability was assessed as described in 8.3.2.

8.3.6 Detection of Interleukin 8 (IL-8) by ELISA

For the quantification of human IL-8 in cell culture supernatants of submerged and ALI mono- and co-cultures treated with HIA bacteria, a human IL-8 ELISA development kit, was purchased from Peprotech EC Ltd. (London, UK). All reagents were part of the kit unless otherwise stated and were prepared according to manufacturer's product information. IL-8 ELISA protocol is described in detail in 2.19.

8.3.7 Statistical analysis

Throughout this chapter statistical analysis of results and significant differences were determined by 1Way-Anova followed byTukey's multiple comparison test. Results are presented as mean ± SD. Results were considered significant when $p \leq 0.05$ = *; $p \leq 0.01$ = **; $p \leq 0.001$ = ***.

8.4 Results

8.4.1 Cell viability and inflammatory response of submerged mono-cultures after exposure to heat-inactivated bacteria (HIA)

8.4.1.1 Exposure of submerged HPF mono-culture to HIA bacteria

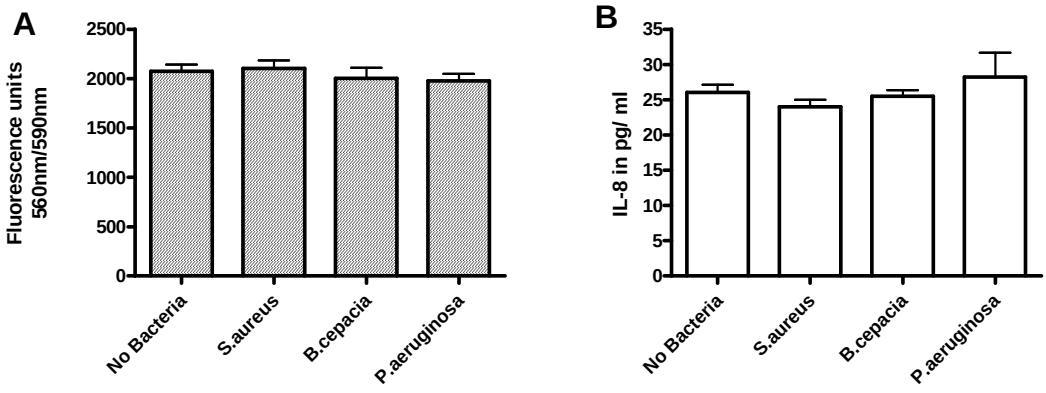

Figure 8.1 Cell viability (A) and IL-8 release (B) of HPF after exposure to HIA bacteria
HPF were grown to confluence in quadruplicate wells of 24-well plate after they were seeded at 1 x 10^5 cells per well. After 24 h medium was changed to serum free medium and another 24 h incubation followed. Afterwards HPF were exposed to approximately 1 x 10^7 cfu/ml of bacterial suspensions for 24 h. Supernatants were collected and analysed for IL-8 concentrations and HPF cell viability was also assessed. For HPF, no significant differences were observed comparing cell viability after exposure to the control cells. Exposure of HPF to HIA *S. aureus, B. cepacia* and *P. aeruginosa* did not cause a significant increase in IL-8 secretion into the supernatant. Data are presented as mean ± SD from 3 individual experiments (n=4).

HPF in submerged mono-cultures were exposed to HIA bacteria for 24 h, apart from control wells, which were exposed to SF-free growth medium only. The bacteria used were *S. aureus, B. cepacia* and *P. aeruginosa* and none of these had an effect on HPF cell viability (figure 8.1 A), when compared to the control baseline fluorescence of 2073.72 ± 68.26 FU. IL-8 concentrations (figure 8.1 B) in collected supernatants were assayed and none of the HIA bacteria induced an IL-8 response by HPF submerged mono-cultures. The supernatants of the control wells had a mean IL-8 concentration of 26.07 ± 1.05 pg/ml.

8.4.1.2 Exposure of submerged C38 mono-culture to HIA bacteria

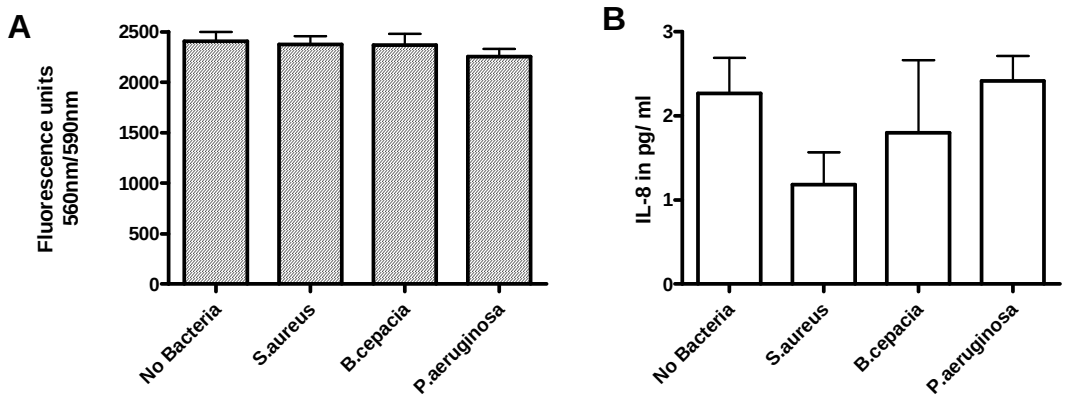

Figure 8.2 Cell viability (A) and IL-8 release (B) of C38 after exposure to HIA bacteria
C38 were seeded onto a 24-well plate at 1 x 10^5 cells per well and incubated for 24 h before they were serum starved for 24 h. Afterwards C38 were exposed to 1 x 10^7 cfu/ml of bacterial suspensions for 24 h. Supernatants were collected and analysed for IL-8 concentrations and C38 were assessed for cell viability. For C38, no significant differences were observed comparing cell viability after exposure to the control (No Bacteria). C38 was exposed to HIA *S. aureus, B. cepacia* and *P. aeruginosa* for 24 h and did not cause a significant difference in IL-8 secretion into the supernatant. Data are presented as mean ± SD from 3 individual experiments (n=4).

Submerged C38 mono-cultures were exposed to HIA *S. aureus*, *B. cepacia* and *P. aeruginosa* for 24 h. Control wells were only exposed to SF-growth medium and were analysed using CTB assay for cell viability (figure 8.2 A), which was 2404.21 ± 95.15 FU at baseline. Cell viability was analysed after the challenges and no significant changes were observed compared to the control. IL-8 secretion (figure 8.2 B) was assayed by ELISA for the presence of IL-8, which was found to unaffected by this exposure to HIA bacteria. IL-8 concentration in control supernatants was 2.27 ± 0.42 pg/ml.

8.4.1.3 Exposure of submerged IB3-1 mono-culture to HIA bacteria

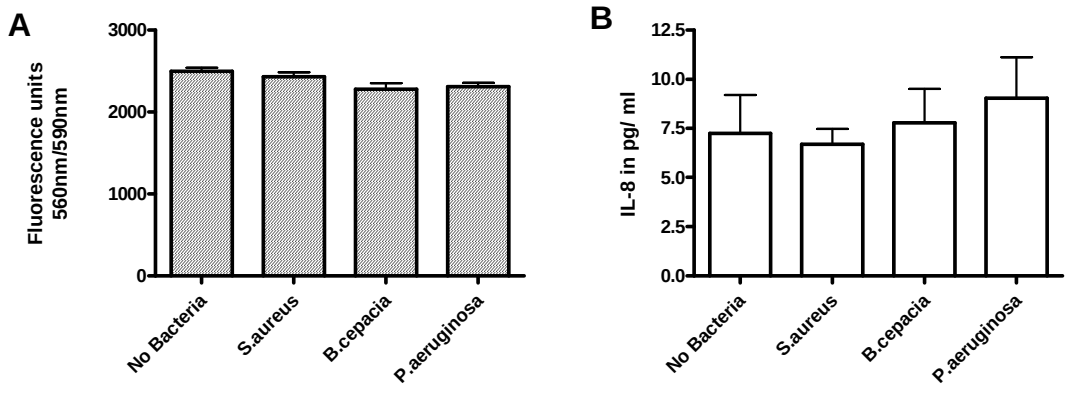

Figure 8.3 Cell viability (A) and IL-8 release (B) of submerged cultures of IB3-1 after exposure to HIA bacteria
IB3-1 were seeded onto a 24-well plate at a cell density of 1 x 10^5 per well and incubated for 24 h before they were serum starved for 24 h. IB3-1 were then exposed to 1 x 10^7 cfu/ml of each bacterium (HIA) for 24 h. Supernatants were collected and analysed for IL-8 concentrations (B). Cell viability was assessed and no significant decrease of cell viability could be identified. IL-8 release into the supernatant was analysed and was found to not be significantly increased for any of these bacteria. Data are presented as mean ± SD from 3 individual experiments (n=4).

For IB3-1 mono-cultures the same challenges were carried out and HIA *S. aureus*, *B. cepacia* and *P. aeruginosa* were added to target wells. Control wells were incubated with SF-growth medium only. IB3-1's cell viability (figure 8.3 A) was not affected by this 24 h challenge and all samples were similar to the measured baseline fluorescence of 2499.49 ± 41.21 FU. IL-8 secretion (figure 8.3 B) of this submerged mono-culture was not induced by any of the HIA bacteria tested. IL-8 concentration of control wells was 7.23 ± 1.97 pg/ml.

8.4.1.4 Exposure of submerged Calu-3 mono-culture to HIA bacteria

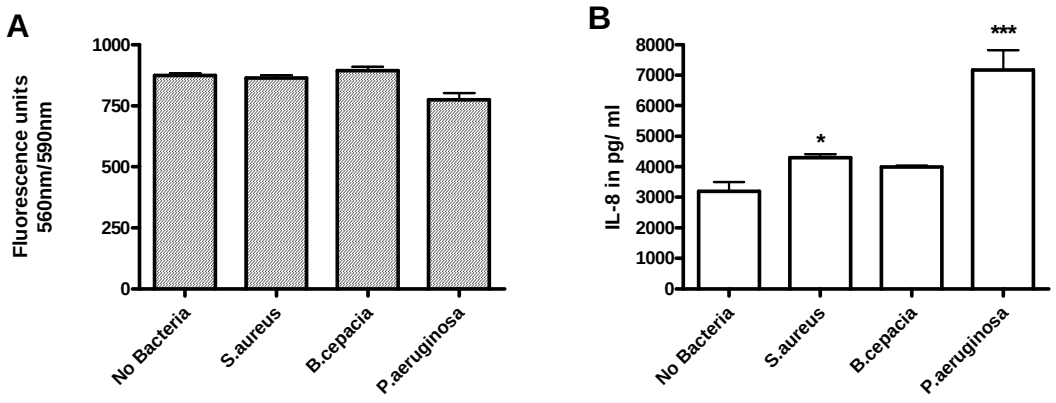

Figure 8.4 Cell viability (A) and Il-8 release (B) of submerged cultures of Calu-3 after exposure to HIA bacteria
Submerged mono-cultures of Calu-3 were seeded onto a 24-well plate at 1 x 10^5 per well and incubated for 24 h before they were serum starved and then exposed to HIA bacteria (1 x 10^7 cfu/ml) for 24 h. Supernatants were collected and analysed for IL-8 concentrations. Cell viability was also assessed but Calu-3 did not show any significant decrease of cell viability after 24 h incubation with these HIA bacteria. IL-8 secretion by Calu-3, however, was significantly increased in response to HIA *S. aureus* or *P. aeruginosa*. Data are presented as mean ± SD from 3 individual experiments (n=4).

Cell viability (figure 8.4 A) of submerged Calu-3 mono-cultures was not influenced by the exposure to HIA *S. aureus*, *B. cepacia* or *P. aeruginosa* for 24 h. At baseline the fluorescent signal measured was 874.47 ± 9.08 FU. IL-8 secretion (figure 8.4 B), however was induced by HIA *S. aureus* and *P. aeruginosa*. The control cells released an IL-8 concentration of 3189.42 ± 306.4 pg/ml, which was increased in response to HIA *S. aureus* to 4290.26 ± 121.23 pg/ml and when challenged with HIA *P. aeruginosa*, the IL-8 concentration was 7166.7 ± 651.69 pg/ml.

8.4.2 Cell viability and IL-8 release of mono- and co-cultures at ALI after exposure HIA bacteria

8.4.2.1 Exposure of submerged HPF mono-culture on TWs to HIA bacteria

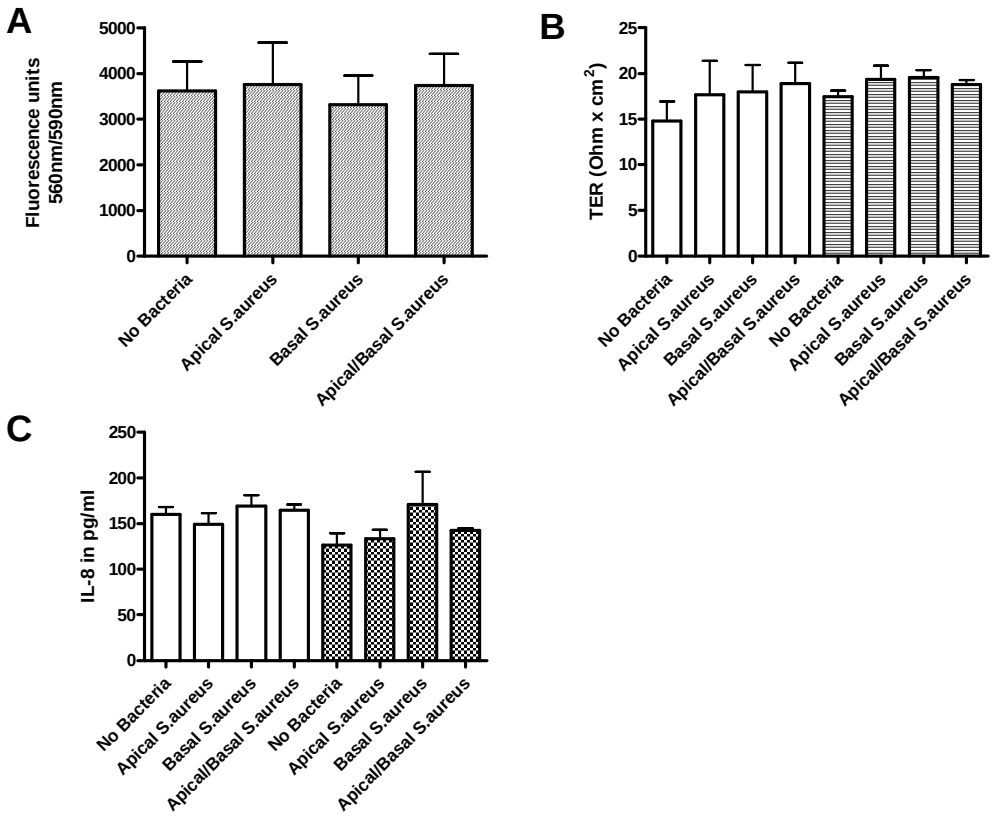

Figure 8.5 Cell viability (A), TER (B) and IL-8 release (C) of HPF after exposure to HIA *S. aureus*
HPF were cultured under submerged conditions on TWs for 14 days before these were challenged with HIA *S. aureus*, which was applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs. Cell viability was not significantly affected, nor was TER measured before (clear bars) and directly after (striped bars) the 24 h incubation with HIA *S. aureus*. No significant increases of IL-8 concentrations were observed for any of the challenges with HIA *S. aureus*. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF were grown on TWs under submerged conditions for 14 days before these were challenged with HIA *S. aureus* from either the apical side, the basolateral side or simultaneously from both sides and were then assessed for cell viability (A), TER (B) of the mono-culture and IL-8 secretions (C) after the 24 h challenge. In figure 8.5 A cell viability is shown for HPF after exposure to *S. aureus* and no significant differences were seen for any of the three challenges when compared to the control, which showed a baseline fluorescence of 3623.43 ± 636.22 FU. Figure 8.5 B represents the results for TER measured before (clear bars) and after (striped bars)

the challenges. TER was not significantly changed over this 24 h incubation with HIA *S. aureus*. TER of control mono-cultures was 14.78 ± 2.16 Ω x cm^2 before and 17.44 ± 0.69 Ω x cm^2 after the challenge. IL-8 concentrations (figure 8.5 C) were measured in apical supernatants (clear bars) and basolateral medium samples (striped bars) collected and secretion was not induced by adding HIA *S. aureus* to either compartment of the TW or to both compartments at the same time. Control cultures, which were exposed to SF-growth medium only had an IL-8 concentration of 159.81 ± 8.56 pg/ml in the apical supernatant and 126.25 ± 13.26 pg/ml in the basolateral medium.

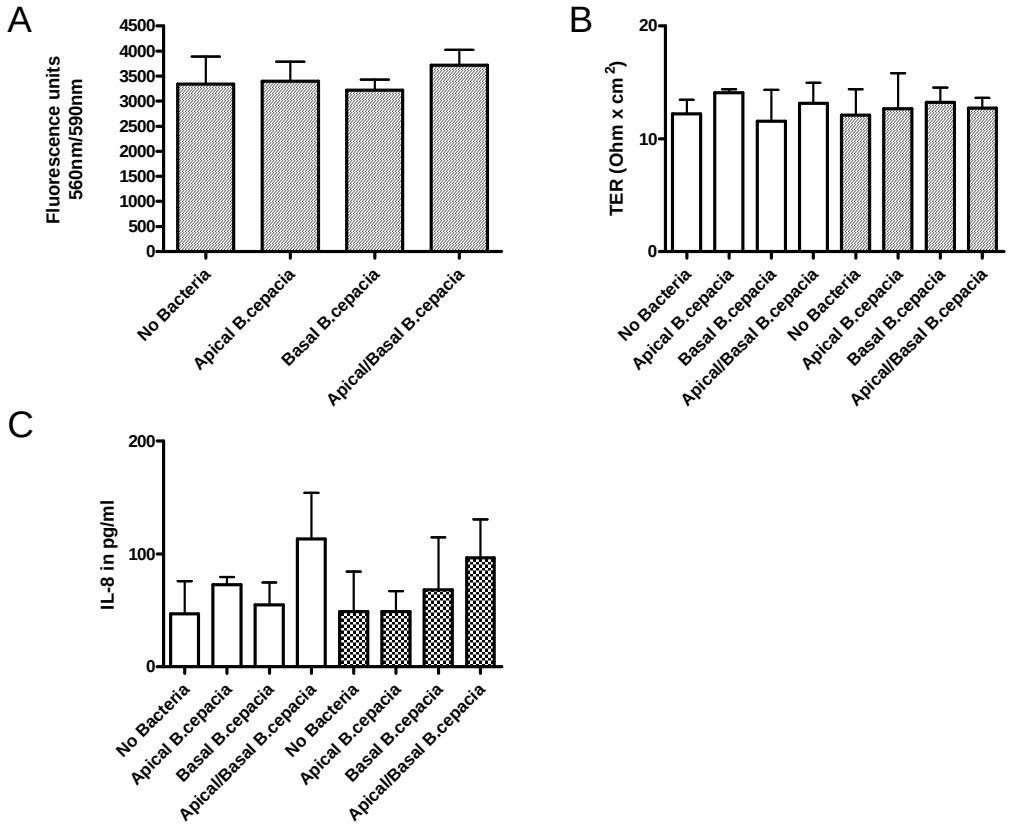

Figure 8.6 Cell viability (A), TER (B) and IL-8 release (C) of HPF after exposure to HIA *B. cepacia*
HPF mono-cultures were grown under submerged conditions on TWs for 14 days before these were challenged with HIA *B. cepacia*. Bacteria were applied either apically, basolaterally or from both sides simultaneously, excluding the control TWs, which were only challenged with growth medium. Cell viability of HPF mono-culture was not affected by the presence of HIA *B. cepacia* for 24 h and nor was the TER, which was measured before (clear bars) and directly after (striped graphs) the 24 h incubation with HIA *B. cepacia*. IL-8 concentrations in apical supernatants (clear bars) and basolateral medium (checked bars) did not show a significant change after any of the challenges compared to the control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF mono-cultures were grown under submerged conditions on TWs for 14 days before they were challenged with HIA *B. cepacia*. The control TWs were treated the same as all others but were exposed to SF-growth medium only. TWs that were challenged were exposed to HIA *B. cepacia* from either the apical side, the basal side or they were exposed to the bacteria on both sides at the same time. HPF's cell viability (figure 8.6 A), which was analysed by CTB was 3340.6 ± 543.59 FU at baseline and was not changed after a 24 h incubation with HIA *B. cepacia*. TER (figure 8.6 B) was measured before (clear bars) and after (striped bars) the challenge. Control TWs showed a TER of 12.21 ± 1.24 Ωcm^2 beforehand and 12.10 ± 2.29 Ωcm^2 afterwards. IL-8 concentrations were analysed in all apical as well as all basolateral medium samples and were found to be unaffected by 24 h exposure to HIA *S. aureus*. The IL-8 concentration (figure 8.6) of the control was 47.02 ± 28.93 pg/ml, when measured in the apical supernatant and it was 48.99 ± 35.56 pg/ml in the basal medium.

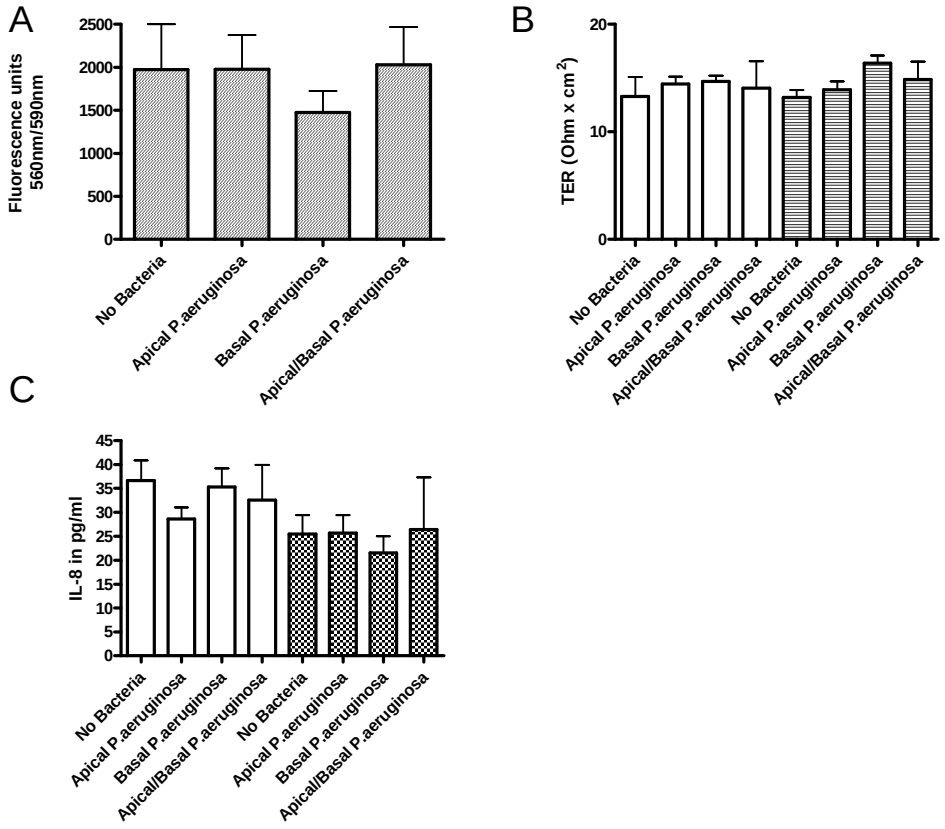

Figure 8.7 Cell viability (A), TER (B) and IL-8 release (C) of HPF mono-culture after exposure to HIA *P. aeruginosa*
HPF mono-cultures were grown under submerged conditions on TWs for 14 days before these cells were exposed to HIA *P. aeruginosa*. Bacteria were applied either apically, basally or simultaneously from both sides. Cell viability of HPF was not significantly changed after 24 h exposure to HIA *P. aeruginosa.* TER was measured before (clear bars) and after (striped bars) the experiment and was stable throughout as no significant difference was observed after 24 h incubation with live *P. aeruginosa*. HPF mono-culture supernatants (clear bars) as well as basolateral medium (checked bars) was analysed for IL-8 concentrations and no significant differences were observed. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

The cell viability (figure 8.7 A) of HPF-monocultures, which were grown under submerged conditions on TWs, was not affected by 24 h exposure to HIA *P. aeruginosa*. The baseline fluorescence measured for the control TWs was 1971.48 ± 528.44 FU. TER (figure 8.7 B) of these mono-cultures was neither affected, when comparing results from before (clear bars) and after (striped bars) the challenges. The control TWs had a TER of 13.27 ± 1.82 Ω x cm^2 before the challenge and 13.20 ± 0.66 Ω x cm^2 after the challenge with HIA *P. aeruginosa*. IL-8 concentrations (figure 8.7 C) were measured in all apical as well as in all basolateral samples collected. The IL-8 secretion was not induced by either apically applied, basolaterally applied or by simultaneous exposure on both sides at the same time. The concentration measured in the

apical supernatant (clear bars) was 36.64 ± 4.21 pg/ml and it was 25.47 ± 3.95 pg/ml in the basal compartment.

8.4.2.2 Exposure of C38 mono-culture at ALI to HIA bacteria

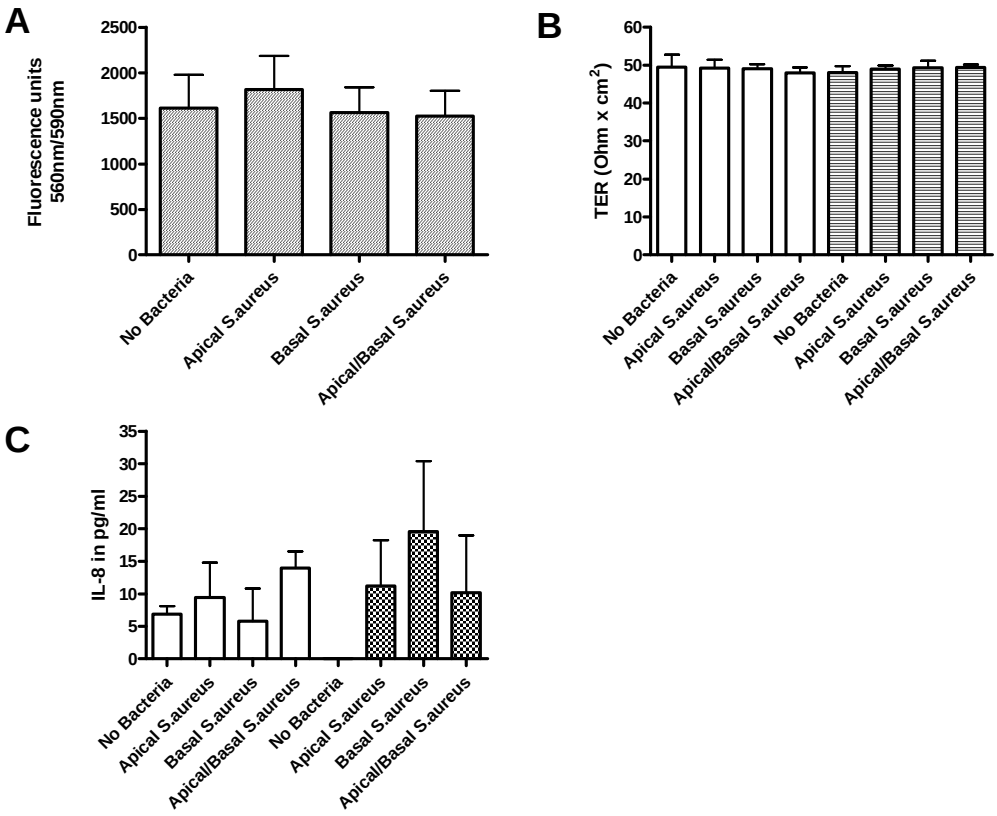

Figure 8.8 Cell viability (A), TER (B) and IL-8 release (C) of C38 grown at ALI after exposure to HIA *S. aureus*
C38 were grown on TWs at ALI for 14 days before they were challenged with HIA *S. aureus*, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were treated with medium only. Cell viability was not significantly changed by this exposure to HIA *S. aureus*. The measured TER before (clear bars) and after (striped bars) the challenges did not show any significant changes. IL-8 concentration was analysed but no differences were noted. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38 mono-cultures grown at ALI were analysed after they were exposed to HIA *S. aureus*. This bacterium did not alter the cell viability (figure 8.8 A), TER (B) or IL-8 (C) concentration in its HIA state. None of the measured parameters were significantly affected. Control cultures of C38 that were exposed to SF-growth medium only showed a fluorescent baseline signal of 1614.75 ± 363.22 FU. TER for these cultures was assessed and was 49.57 ± 3.26 Ω x cm^2 before (clear bars) the challenge and 48.07 ± 1.73 Ω x cm^2 after (striped bars) the bacterial exposure. IL-8 secretion into the apical supernatant (clear bars) of control mono-cultures of C38 was 6.87 ± 1.26 pg/ml, whereas no IL-8 was observed in the basolateral medium sample (checked bars).

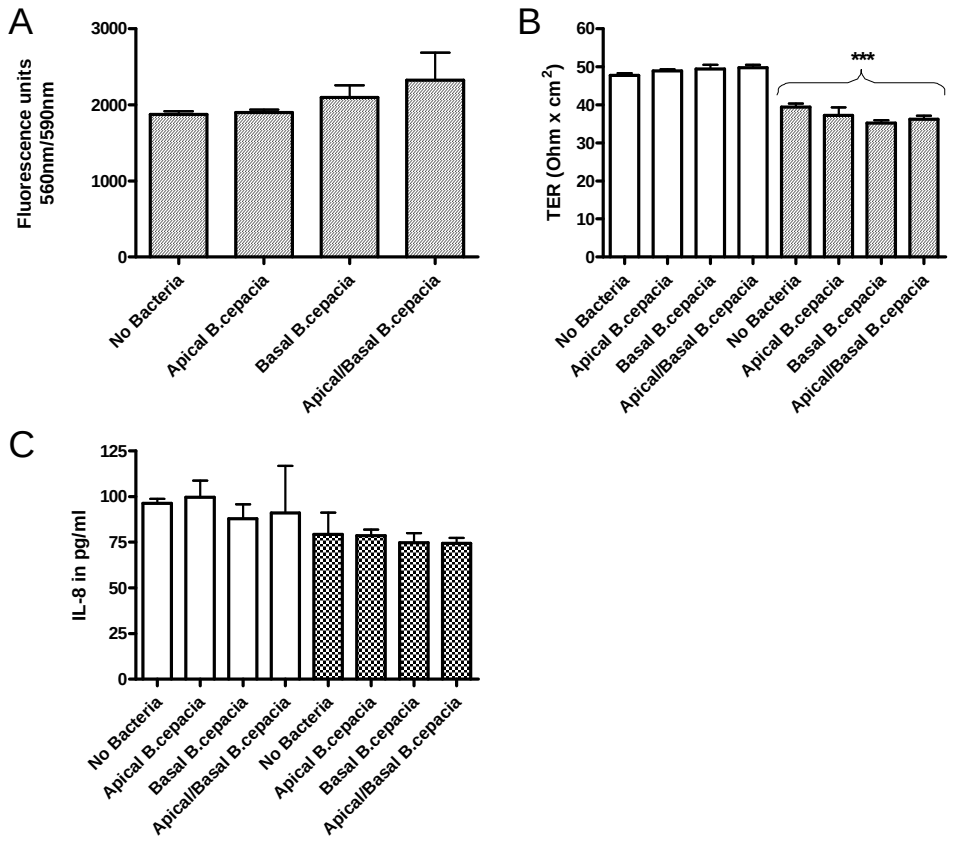

Figure 8.9 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to HIA *B. cepacia*

Mono-cultures of C38 grown at ALI were exposed to HIA *B. cepacia*, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs were only challenged with growth medium and no bacteria. Cell viability (A) and IL-8 release (C) were not affected by the presence of HIA *B. cepacia*. TER after the challenge (striped bars), however, was significantly lower, including the control, comparing to the corresponding TER from before (clear bars) the challenges. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38 mono-cultures grown at ALI for 14 days before they were challenged with HIA *B. cepacia*, which was either applied apically, basolaterally or from both sides at the same time. Cell viability (figure 8.9 A) was not affected after 24 h incubation and all challenged TWs had a similar fluorescent signal from CTB as the control (1875.07 ± 40.95 FU). TER (figure 8.9 B) of this mono-culture was measured beforehand (clear bars) and straight afterwards (striped bars), which was significantly decreased for all TWs measured, including the control. In order, the control was 47.74 ±0.58 Ω x cm^2 before the challenge and 39.49 ± 0.85 Ω x cm^2 afterwards. Apically challenged TWs had a TER of 48.95 ± 0.39 Ω x cm^2 beforehand and 37.25 ± 2.06 Ω x cm^2 were

measured afterwards. When TWs were challenged basolaterally these values were 49.43 ± 1.12 Ω x cm^2 before and 35.2 ± 0.76 Ω x cm^2 after the exposure to *B. cepacia*. IL-8 secretion (figure 8.9 C) was not induced by HIA *B. cepacia* and was 96.35 ± 2.43 pg/ml in the control's apical supernatant and 79.47 ± 11.83 pg/ml in the control's basolateral medium.

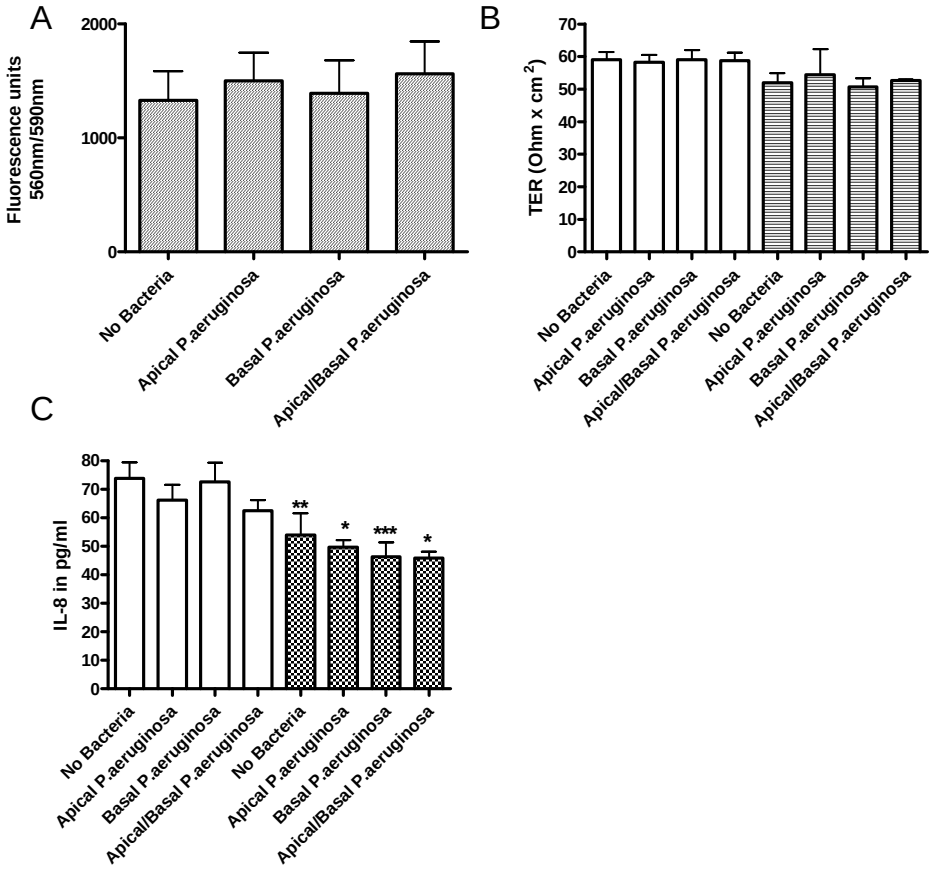

Figure 8.10 Cell viability (A), TER (B) and IL-8 release (C) of C38 mono-cultures after exposure to HIA *P. aeruginosa*
C38 mono-cultures were grown at ALI for 14 days and were then exposed to HIA *P. aeruginosa*, which was applied either apically, basolaterally or from both sides simultaneously. Control TWs, which were treated the same but without bacteria served as control. Neither cell viability (A) of C38, nor the TER was significantly changed by any of these challenges. IL-8 concentrations in the apical supernatants (clear bars) were not found to be changed after 24 h exposure to HIA *P. aeruginosa.* The basolateral medium (checked bars) showed in all cases, including the control, significantly lower IL-8 compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

C38-mono-cultures were grown at ALI for 14 days before these were challenged with HIA *P. aeruginosa*, either apically, basolaterally or from both sides at the same time. Cell viability (figure 8.10 A) was not significantly affected by this challenge. Baseline fluorescence measured

for the control was 1328.78 ± 257.56 FU. The electrical resistance was monitored by measuring the TER (figure 8.10 B) before (clear bars) and after (striped bars) the challenges and was found to be stable throughout the experiment. TER measured for control TWs was 59.03 ± 2.44 Ω x cm^2 before and 51.88 ± 3.05 Ω x cm^2 after the exposure to HIA *P. aeruginosa*. IL-8 concentrations (figure 8.10 C) were assayed for apical supernatants (clear bars) as well as for basolaterally collected medium (checked bars). These were not significantly different to the analysed controls, which were 73.921 ± 5.52 pg/ml in the apical supernatant and 54.05 ± 7.59 pg/ml in the basolateral sample. All basolateral samples, however, were significantly lower compared to their corresponding apical supernatants. For the apical challenged TWs this was 66.25 ± 5.28 pg/ml on the apical side and IL-8 concentration was 49.69 ± 2.49 pg/ml on the basolateral side of the mono-culture. Basally challenged TWs had 72.60 ± 6.73 pg/ml in the apical supernatant compared to 46.28 ± 5.06 pg/ml in the basolateral medium. When C38 mono-cultures were challenged simultaneously from both sides, the IL-8 concentration found apically was 62.57 ± 3.73 pg/ml and it was 45.81 ± 2.35 pg/ml in the basal medium collected.

8.4.2.3 Exposure of IB3-1 mono-cultures at ALI to HIA bacteria

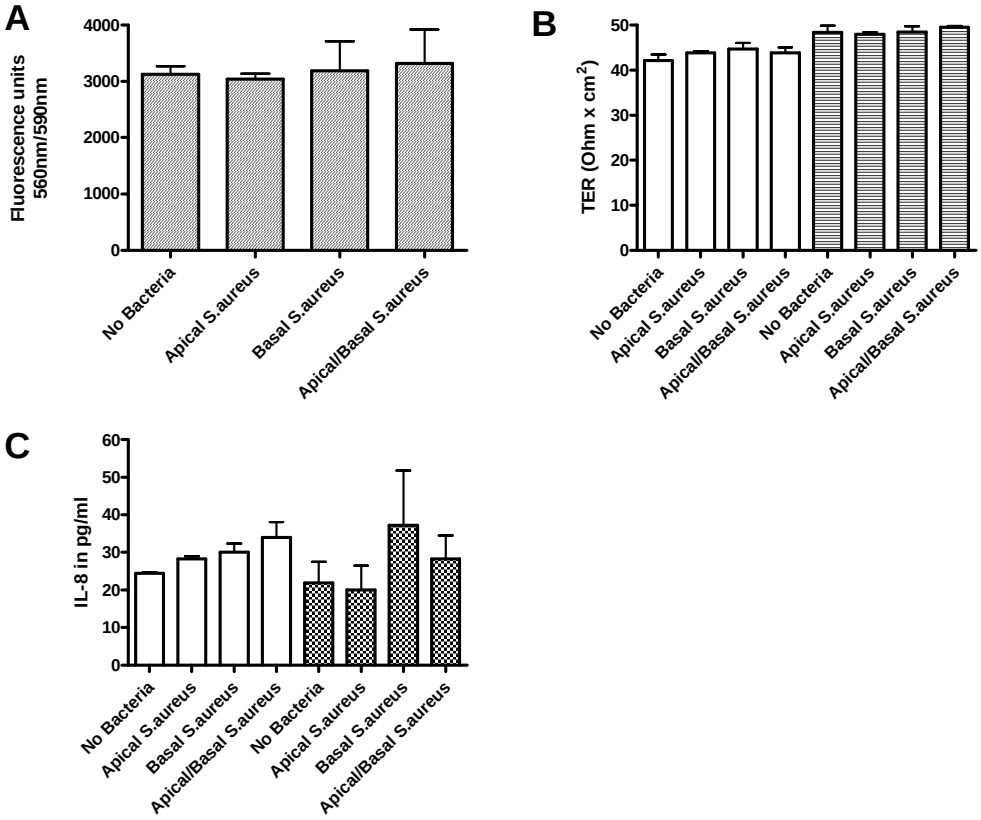

Figure 8.11 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 grown at ALI after exposure to HIA *S. aureus*
IB3-1 were cultured at ALI for 14 days before they were challenged with HIA *S. aureus*. Bacteria were applied either apically, basolaterally or from both sides at the same time and the control TWs were only treated with medium. For IB3-1 no significant changes were observed looking at cell viability and when comparing TER from before (clear bars) and after (striped bars). IL-8 secretions were not increased significantly by this bacterium in the apical supernatant or in the basolateral medium analysed. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

IB3-1 mono-culture were grown at ALI for 14 days before these were challenged with HIA *S. aureus*, which was applied either apically, basolaterally or apically/ basolaterally at the same time. After 24 h exposure cell viability (figure 8.11 A) of this mono-culture was analysed and was found to be unaffected by this event. The baseline fluorescence of the control was 3125.13 ± 147.00 FU. TER (figure 8.11 B) was measured before (clear bars) and after (striped bars) the challenge with no significant difference after any of the challenges when compared to the control, which showed a TER of 42.17 ± 1.31 Ω x cm^2 beforehand and 48.40 ± 1.48 Ω x cm^2. IL-8 secretion (figure 8.11 C) was analysed in apical supernatants (clear bars) and basolateral medium

samples (checked bars) but no increase was observed for any of the challenges compared to the control (beforehand: 24.49 ± 0.26 pg/ml, afterwards: 21.87 ± 5.59 pg/ml).

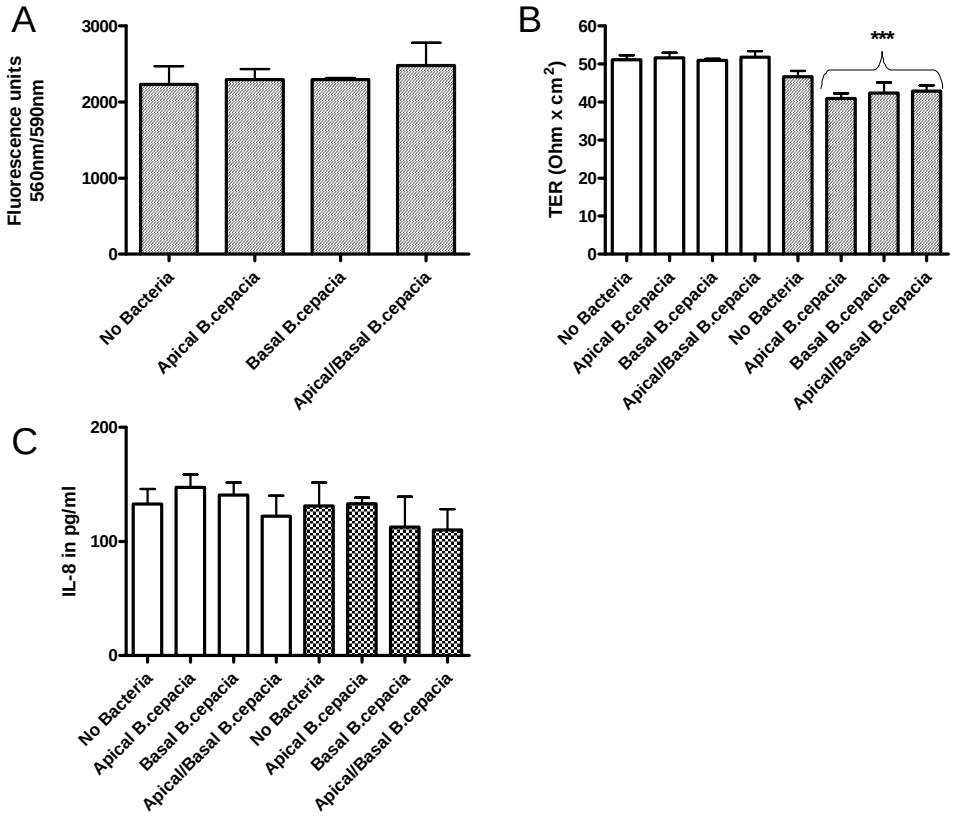

Figure 8.12 Cell viability (A), TER (B) and IL-8 release (C) after exposure of IB3-1 to HIA *B. cepacia* of IB3-1 grown at ALI

IB3-1 mono-cultures were challenged with HIA *B. cepacia*. TWs were either challenged apically, basolaterally or from both sides simultaneously. IB3-1 control TWs were treated the same way as all other TWs but with medium only. In this case cell viability was not significantly changed by the exposure to HIA bacteria. TER was measured before (clear bars) and was significantly decreased for all three challenges after 24 h (striped bars) incubation. IL-8 concentrations observed in apical supernatants (clear bars) as well as in basolateral medium samples (checked bars) were not significantly changed by HIA *B. cepacia.* Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

IB3-1 mono-cultures grown at ALI were exposed to HIA *B. cepacia*. The HIA bacteria were added either to the apical side, to the basolateral side or to both sides simultaneously. Cell viability (figure 8.12 A) was measured straight after 24 h incubation and was found to be unaffected (control: 2230.26 ± 242.62 FU). TER (figure 8.12 B) measurements showed that HIA bacteria significantly decrease this mono-cultures electrical resistance. After all three challenges the TER was found to be significantly decreased, when compared to their corresponding TER from

before. The control TWs showed a TER of 51.11 ± 1.20 Ω x cm^2 beforehand and 46.67 ± 1.52 Ω x cm^2 afterwards. When IB3-1 were challenged from the apical side the TER fell from 51.66 ± 1.32 Ω x cm^2 down to 40.99 ± 1.29 Ω x cm^2. Similarly readings were observed for basally challenged TWs as these had a TER of 50.93 ± 0.50 Ω x cm^2 before the exposure to HIA *B. cepacia*, which then decreased to 42.35 ± 2.85 Ω x cm^2 after 24 h incubation. IL-8 concentrations (figure 8.12 C) were not changed by any of these challenges and stayed roughly around the same concentrations as the control, which was 132.87 ± 13.01 pg/ml in the apical supernatants (clear bars) and 131.27 ± 20.46 pg/ml in the basolateral medium samples (checked bars).

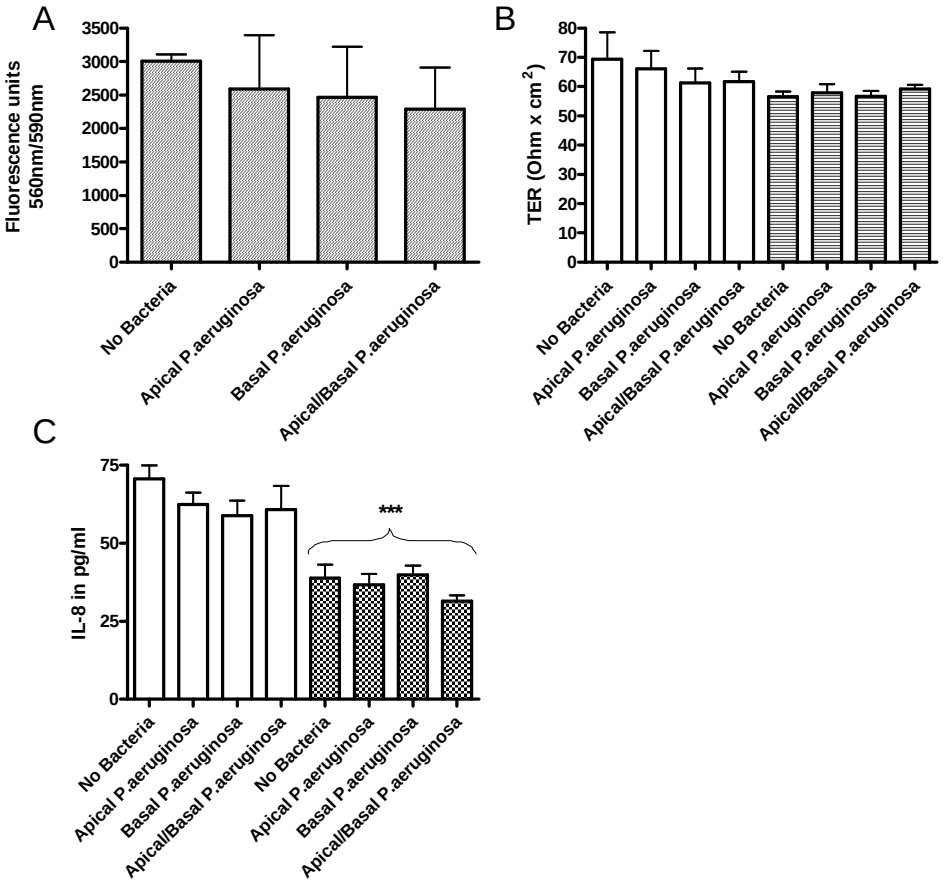

Figure 8.13 Cell viability (A), TER (B) and IL-8 release (C) of IB3-1 mono-culture after exposure to HIA *P. aeruginosa*

IB3-1 mono-cultures were grown at ALI for 14 days before these cells were exposed to HIA *P. aeruginosa*, which were applied either apically, basally or simultaneously from both sides. Cell viability after 24 h exposure to HIA bacteria was not significantly decreased for any of the challenges compared to the control. TER was measured before (clear bars) and after (striped bars) the experiment but no significant difference was observed after 24 h incubation with HIA *P. aeruginosa*. IL-8 release of IB3-1 mono-culture was not changed either but one interesting aspect to mention is significantly lower IL-8 concentrations in all basolateral media samples (checked bars) compared to their corresponding apical supernatant (clear bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Mono-cultures of IB3-1 were challenged with HIA *P. aeruginosa*, which were applied apically, basolaterally or simultaneously from both sides for 24 h. Cell viability (figure 8.13 A) was investigated after these challenges and no significant differences were observed. Baseline fluorescence of this mono-culture was 3006.96 ± 98.67 FU. TER (figure 8.13 B) was measured before and after the 24 h incubation with HIA *P. aeruginosa*, which was not affected. The control TWs had a TER of 69.37 ± 9.10 Ω x cm^2 before the challenge and 56.50 ± 1.84 Ω x cm^2 afterwards. IL-8 concentrations (figure 8.13 C) were measured, and in the control TWs, were 70.59 ±4.36 pg/ml apically and 38.92 ± 4.31 pg/ml basolaterally. No significant inductions of IL-8 secretions were observed but again all basolateral samples were significantly lower in IL-8 compared to their corresponding apical samples. Apically challenged cells secreted 62.38 ± 3.87 pg/ml IL-8 to the apical supernatant and 36.67 ± 3.49 pg/ml to the basolateral medium. When IB3-1 mono-cultures were challenged with HIA *P. aeruginosa* in the basal compartment the measured IL-8 concentration was 58.83 ± 4.82 pg/ml apically and 39.93 ± 2.93 pg/ml basolaterally. The simultaneous challenge from both sides resulted in 60.86 ±7.61 pg/ml apical IL-8 and in 31.46 ± 1.79 pg/ml basal IL-8.

8.4.2.4 Exposure of Calu-3 mono-cultures at ALI to HIA bacteria

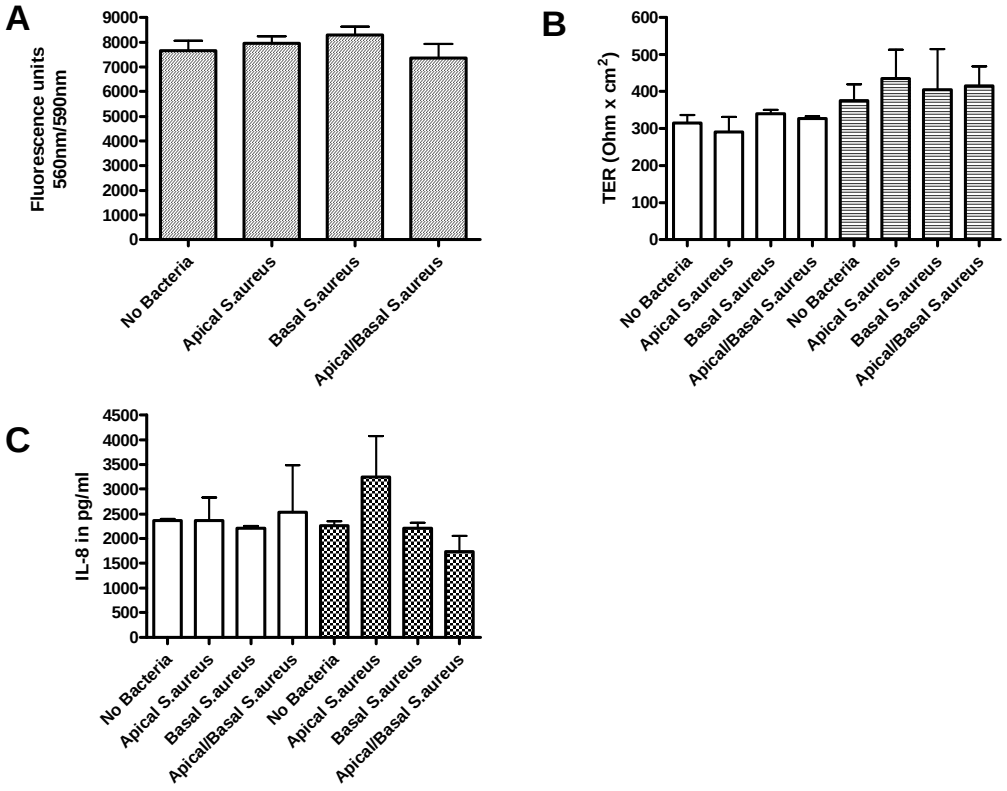

Figure 8.14 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 at ALI after exposure to HIA *S. aureus* Calu-3 mono-culture were grown at ALI for 14 days before these cells were challenged with HIA *S. aureus.* Bacteria were applied either apically, basolaterally or on both sides at the same time. Neither Calu-3's cell viability was affected by this 24 h exposure, nor was the TER, which was measured before (clear bars) and after (striped bars) the challenge. IL-8 secretion of Calu-3 at ALI was not significantly different to control either in apical supernatants (clear bars) or basolateral medium (checked bars) after any of the challenges. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3's cell viability (figure 8.14 A) of the control TWs was 7660.61 ± 394.54 FU at baseline, which was similar to cell viability found after every challenge. TER (figure 8.14 B) measurements were taken before (clear bars) and after (striped bars) the challenges and were unaffected by this challenge with HIA *S. aureus* for 24 h. The control TWs had an electrical resistance of 315.77 ± 21.47 Ωcm^2 before the challenge and 375.72 ± 43.98 Ωcm^2 after the challenge. IL-8 secretion (figure 8.14 C) was not induced by any of the challenges using HIA *S. aureus.* The baseline IL-8 secretion was 2368.60 ± 30.06 pg/ml in the apical supernatant and 2259.32 ± 98.87 pg/ml.

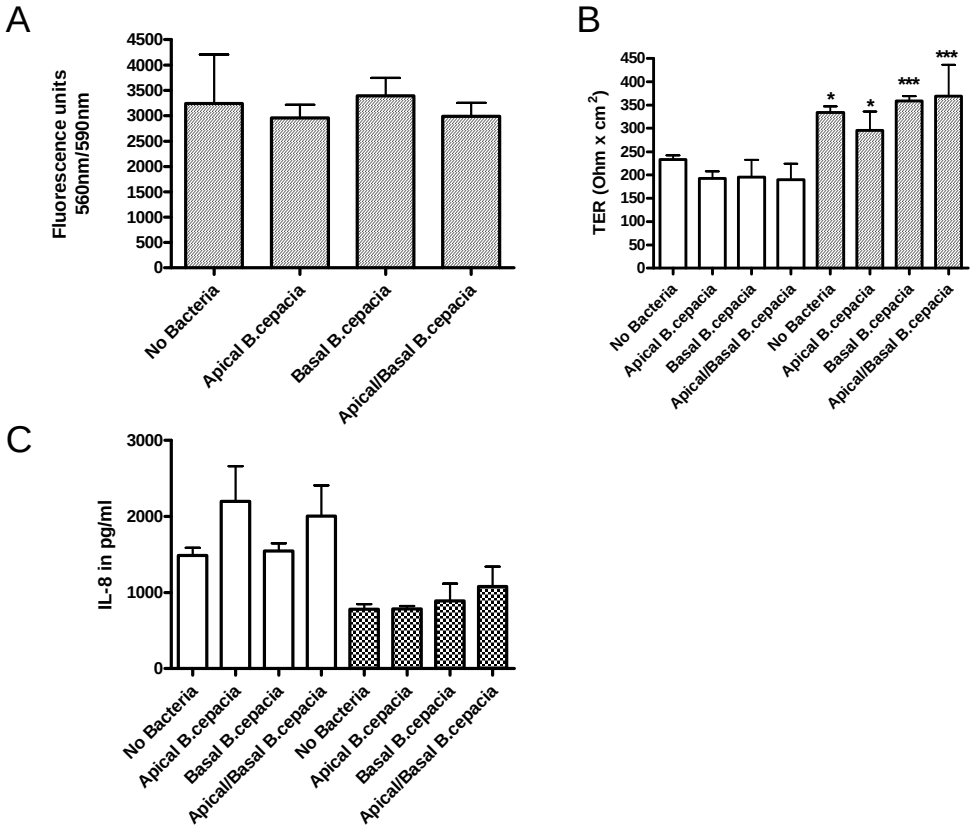

Figure 8.15 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 after exposure to HIA *B. cepacia*
Mono-cultures of Calu-3 were at ALI for 14 days before they were challenged with HIA *B. cepacia,* either apically, basolaterally or from both sides at the same time after. Cell viability of this mono-culture was not significantly changed throughout this bacterial challenge. TER was measured before (clear bars) and after (striped bars) the challenges and was found to be significantly higher, including the control after the 24 h challenge. IL-8 concentration in the apical supernatants (clear bars) was not significantly increased when compared to the control. An aspect to be noted is the significantly lower IL-8 concentration in the basolateral medium (checked bars) of apically challenged and apically/basolaterally challenged TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3 mono-cultures at ALI were challenged with HIA *B. cepacia* after they were cultured for 14 days. Bacteria were added either to the apical compartment, to the basal compartment or to both at the same time. Cell viability (figure 8.15 A) was measured after the 24 h challenge and was not affected by this event. 3243.40 ± 962.09 FU were measured for the control TWs and the challenged TWs were not significantly different. TER (figure 8.15 B) for this mono-culture was assayed before (clear bars) the challenge and after (striped bars) the challenge. TER from before the challenges were 233.61 ± 8.31 Ω x $cm^2$for the control, 192.87 ± 14.6 Ω x cm^2 for the apical challenges TWs, 195.16 ± 37.01 Ω x cm^2 for the basally challenged TWs and 189.56 ± 34.72 Ω x cm^2, when challenged from both sides. After the challenges these were found to be significantly higher compared to their corresponding TER from before. The results were 333.92 ± 13.04 Ω x

cm^2 for the control, 295.64 ± 40.12 Ω x cm^2 for the apical challenged TWs, 358.67 ± 10.64 Ω x cm^2, for the basally challenged and 368.94 ± 67.35 Ω x cm^2, when TWs were challenged from both sides at the same time. IL-8 secretion (figure 8.15 C) in apical supernatants and basolateral medium samples were analysed and were not significantly increased by this challenge. IL-8 concentration in the apical supernatant of the control was 1485.88 ± 103.98 pg/ml and it was 779.08 ± 67.36 pg/ml in the basolateral control.

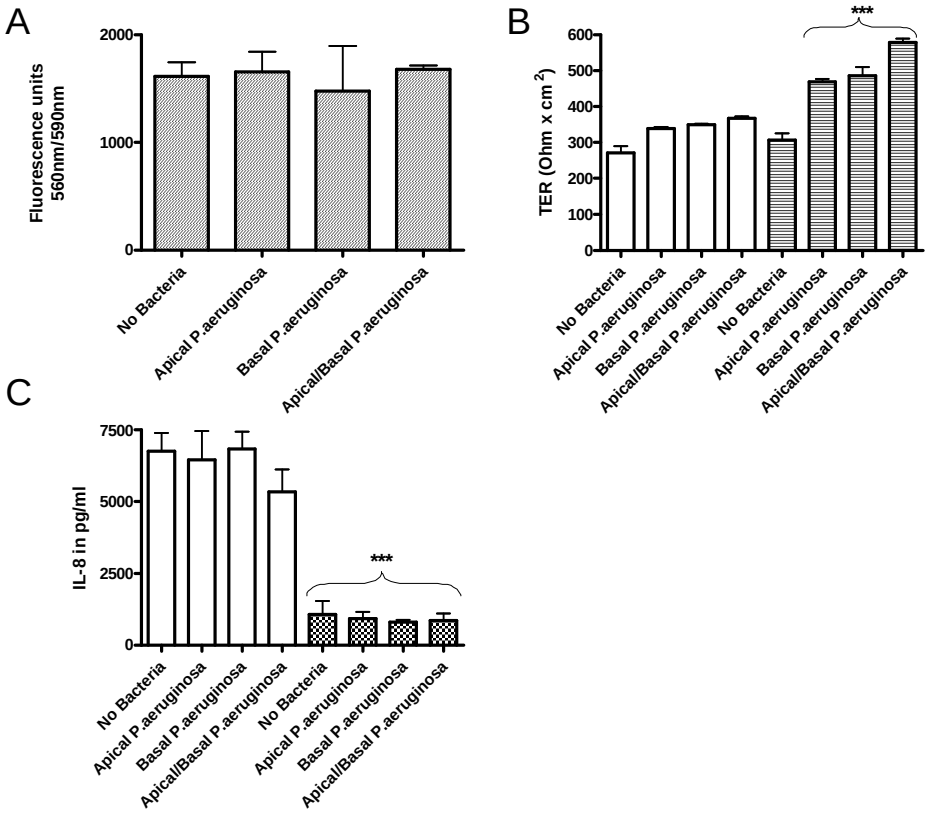

Figure 8.16 Cell viability (A), TER (B) and IL-8 release (C) of Calu-3 mono-culture after exposure to HIA *P. aeruginosa*
Calu-3 mono-cultures at ALI were challenged with HIA *P. aeruginosa,* either apically, basolaterally or both at the same time after 14 days of culture at ALI. Cell viability was not significantly decreased after any of these challenges. TER was measured before (clear bars) and after (striped bars) the challenges and was significantly increased after all three challenges with HIA *P. aeruginosa*. IL-8 concentration in the apical supernatants (clear bars) were all at a similar level and so were the basolateral samples (checked bars), which were all significantly lower in IL-8 than their corresponding apical supernatants. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Calu-3 mono-cultures were grown at ALI for 14 days before they were challenged with HIA *P. aeruginosa* for 24 h. HIA bacteria were added to the apical side, basal side or on both sides at

the same time. Calu-3's cell viability (figure 8.16 A) was not affected by these HIA bacteria and the baseline fluorescence measured for the control was 1613.77 ± 128.51 FU. TER (figure 8.16 B) was found to be significantly increased after all three challenges, which was not observed for the control. TER of the control TWs was 271.44 ± 18.42 beforehand and 306.68 ± 18.54 afterwards. Apically challenged TWs showed an increase from 339.31 ± 4.40 Ω x cm^2 before to 469.00 ± 8.02 Ω x cm^2 after the challenge. When Calu-3 were challenged basolaterally the TER afterwards was 486.12 ± 24.03 (before 349.87 ± 2.56 Ω x cm^2) and when challenged from both sides the TER resulted in 579.48 ± 10.40 (before 367.51 ± 5.82 Ω x cm^2). IL-8 concentrations (figure 8.16 C) were found to be unaffected by this exposure. Basolateral samples, including the control were significantly lower than their corresponding apical sample. In control TWs there was 6751.17 ± 634.90 pg/ml of IL-8 in the apical supernatant compared to 1071.96 ± 465.68 pg/ml found basolaterally. When TWs were challenged apically, 6452.28 ± 1004.17 pg/ml was found in the apical sample and 924.23 ± 242.56 pg/ml was found in the basal medium. Calu-3 mono-cultures were also exposed to HIA *P. aeruginosa* from the basolateral side and 6837.03 ± 594.22 pg/ml of IL-8 were found in the apical supernatant compared to 806.19 ± 74.75 pg/ml IL-8 in the basolateral sample. Challenging the mono-culture from both sides simultaneously resulted in 5331.49 ± 785.41 pg/ml in the apical supernatant and in 858.93 ± 243.65 pg/ml in the basal medium analysed.

8.4.2.5 Exposure of HPF-C38 co-cultures to HIA bacteria

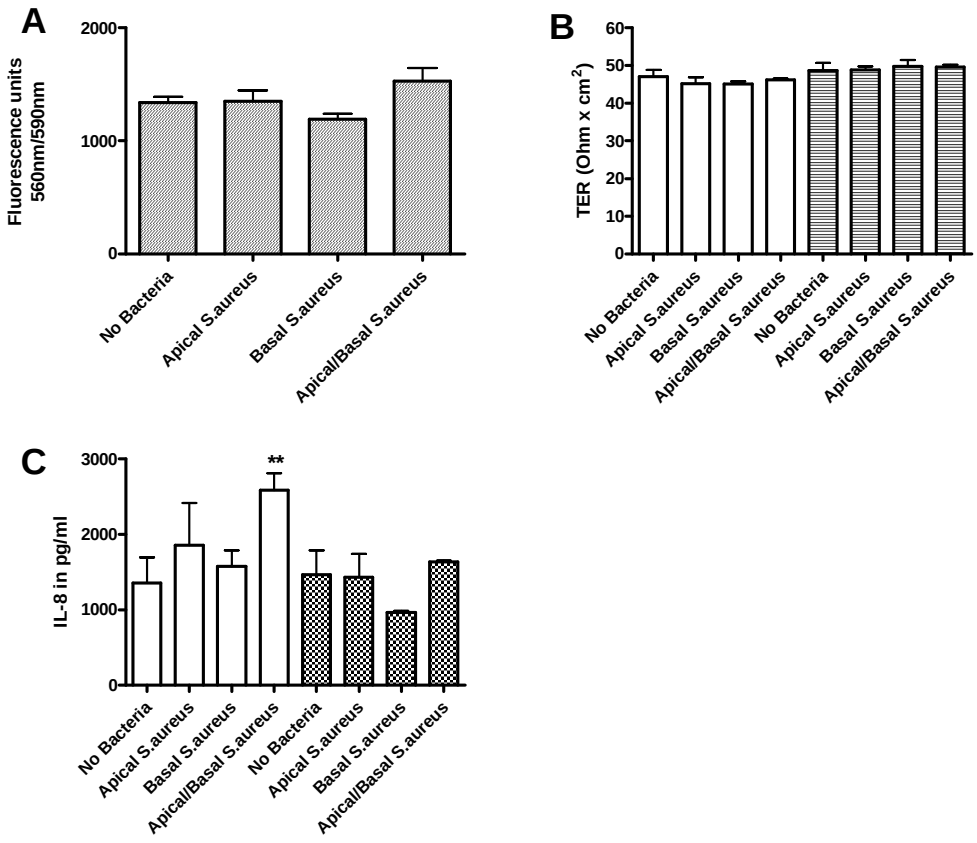

Figure 8.17 Cell viability (A), TER (B) and IL-8 release (C) after exposure to HIA *S. aureus* of HPF-C38 grown at ALI
After 14 days at ALI HPF-C38 were challenged with HIA *S. aureus*, which was applied either apically, basolaterally or from both sides simultaneously apart from the control TWs, which were challenged with medium only. Cell viability of this co-culture was not significantly affected after 24 h exposure to HIA *S. aureus* compared to the control. The measured TER after the challenges (striped bars) was not different when compared to their corresponding TER measured before (clear bars). IL-8 secretion was only significantly increased in the apical supernatant (clear bars) by simultaneous treatment with HIA *S. aureus* from apical and basolateral side. This increase was not observed for basolateral medium samples (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-C38 co-cultures were grown on TWs for 14 days before these were exposed to HIA *S. aureus*, which were applied either apically, basolaterally or from both sides at the same time. Cell viability (figure 8.17 A) was assessed after the 24 h incubation and was found to be unaffected compared to the baseline fluorescence of the control (1341.06 ± 48.15 FU). TER (figure 8.17 B) of this mono-culture system was stable throughout the experiment and measured 47.08 ± 1.81 for the control before the challenge and 48.62 ± 2.12 after the challenge. IL-8 secretions (figure 8.18 C) were only significantly increased in apical supernatants (clear bars) by HIA *S. aureus*, when the bacteria were applied simultaneously on both sides. In this case IL-8 was

2587.65 ± 223.37 pg/ml compared to 1355.34 ± 337.57 pg/ml in the apical supernatant of the control.

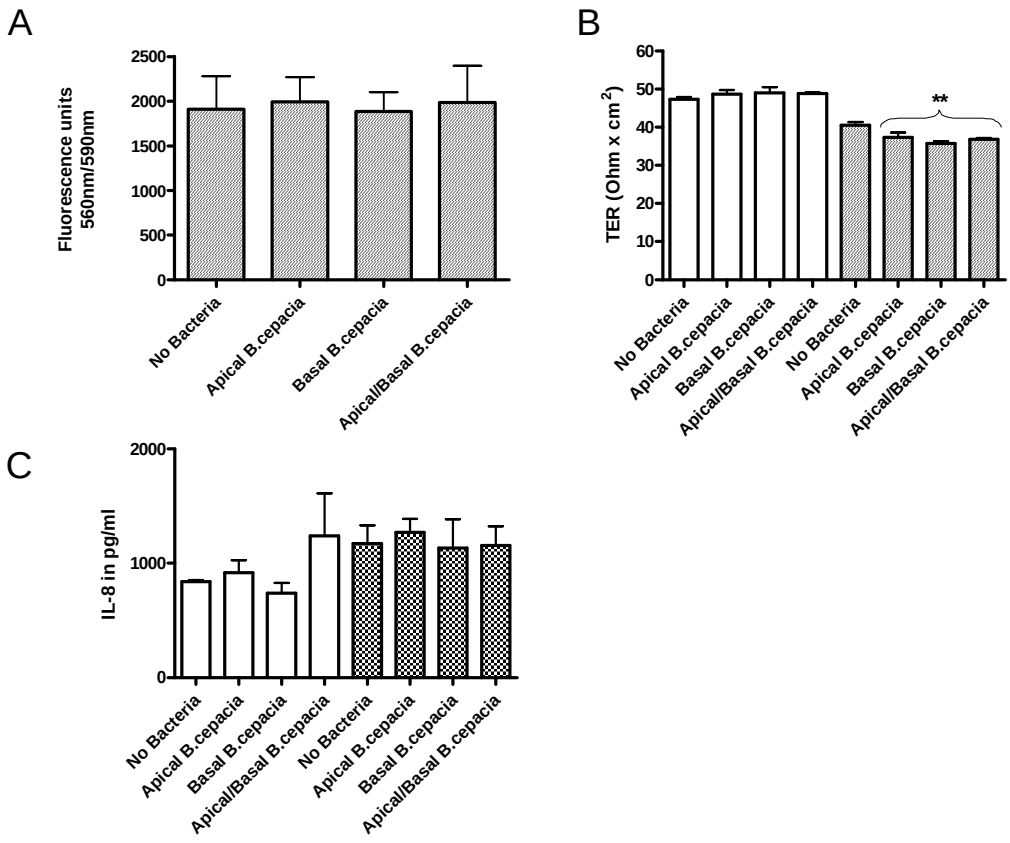

Figure 8.18 Cell viability (A), TER (B) and IL-8 release (C) after exposure to HIA *B. cepacia* of HPF-C38 co-culture grown at ALI
HPF-C38 co-culture TWs were challenged with HIA *B. cepacia*, which were applied to either the apical side of the culture, to the basolateral side or to both sides at the same time. HPF-C38 controls were only challenged using medium without bacteria. In this case cell viability was not affected by HIA *B. cepacia.* However, the TER measured before (clear bars) and after (striped bars) the challenges was observed to be significantly lower after all three challenges. IL-8 concentrations were analysed and none of the apical supernatants (clear bars) and none of the basolateral media samples (checked bars) were found to be significantly changed. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-C38 co-culture was grown at ALI for 14 days before these were exposed to HIA *B. cepacia*, which was added either to the apical side, to the basal side or to both sides at the same time. Cell viability (figure 8.18 A) was observed after 24 h exposure to HIA *B. cepacia* and was not found to be significantly changed by this event. The baseline fluorescence of the control was 1912.06 ± 365.94 FU. TER (figure 8.18 B) of this co-culture was measured before (clear bars) and after (striped bars) this event and was observed to be significantly decreased after the 24 h incubation with HIA *B. cepacia*. The control TWs TER was 47.34 ± 0.63 Ω x cm^2 before the

challenge and slightly lower with 40.52 ± 0.85 Ω x cm^2 after the challenge. When this co-culture was exposed to bacteria apically the TER fell from 48.69 ± 1.07 Ω x cm^2 down to 37.36 ± 1.24 Ω x cm^2. Exposure from the basal compartment caused a similar effect and TER was 35.79 ± 0.55 Ω x cm^2 afterwards compared to 49.06 ± 1.46 Ω x cm^2 beforehand. Simultaneous challenge from both sides of the co-culture decreased TER from 48.88 ± 0.23 Ω x cm^2 down to 36.81 ± 0.35 Ω x cm^2. IL-8 secretion (figure 8.18 C) by this co-culture was not significantly affected by this HIA bacterium. In the apical supernatant (clear bars) of the control there was 812.14 ± 61.23 pg/ml and in the basal medium there was 879.69 ± 248.81 pg/ml.

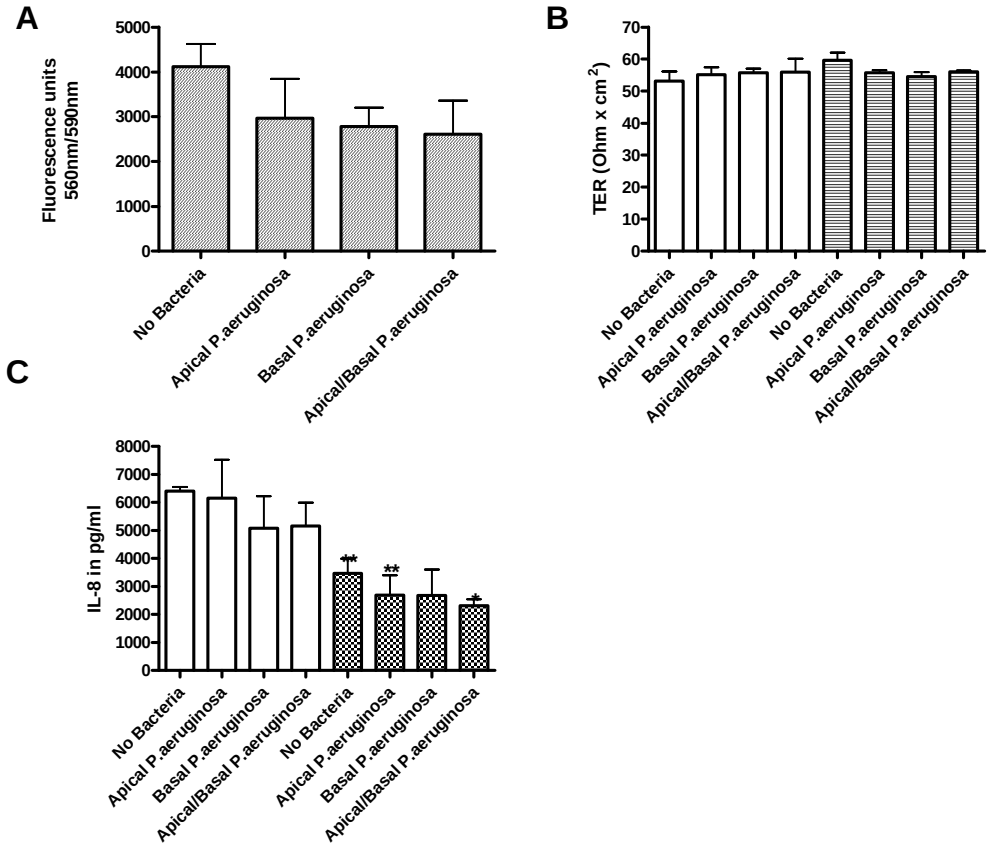

Figure 8.19 Cell viability (A), TER (B) and IL-8 release (C) after exposure to HIA *P. aeruginosa* of HPF-C38 grown at ALI

HPF-C38 were grown at ALI for 14 days before HIA *P. aeruginosa* were applied were either apically, basolaterally or from both sides simultaneously. HPF-C38 control was challenged with medium only. Cell viability was not significantly decreased but showed a trend toward it compared to the control. The measured TER before (clear bars) and after the challenges (striped bars) was not changed by this 24 h incubation with HIA *P. aeruginosa*. IL-8 concentrations in apical supernatants (clear bars) as well as in the basolateral media samples (checked bars) stayed roughly at the same levels but concentrations were significantly lower in basolateral samples when compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-C38 co-cultures that were challenged with HIA *P. aeruginosa* after 14 days at ALI did not show any significant change in cell viability (figure 8.19 A) after a 24 h exposure. The baseline fluorescene observed for this co-culture was 4113.83 ± 509.10 FU. TER (figure 8.19 B) was stable throughout the experiment and before (clear bars) the challenges the control showed a TER of 53.13 ± 3.02 Ω x cm^2 and afterwards it was 59.62 ± 2.43 Ω x cm^2. IL-8 secretions (figure 8.19 C) were measured in the apical supernatants (clear bars) as well as in the basolateral medium samples (checked bars) and was not significantly changed by the exposure to HIA *P. aeruginosa* but all basolateral medium samples, apart from basally challenged ones, had significantly less IL-8 compared to their corresponding apical supernatant. The control had a concentration of 6409.29 ± 134.69 pg/ml in the apical supernatant compared to 3460.45 ± 529.41 pg/ml in the basal medium samples analysed.

8.4.2.6 Exposure of HPF-IB3-1 co-culture to HIA bacteria

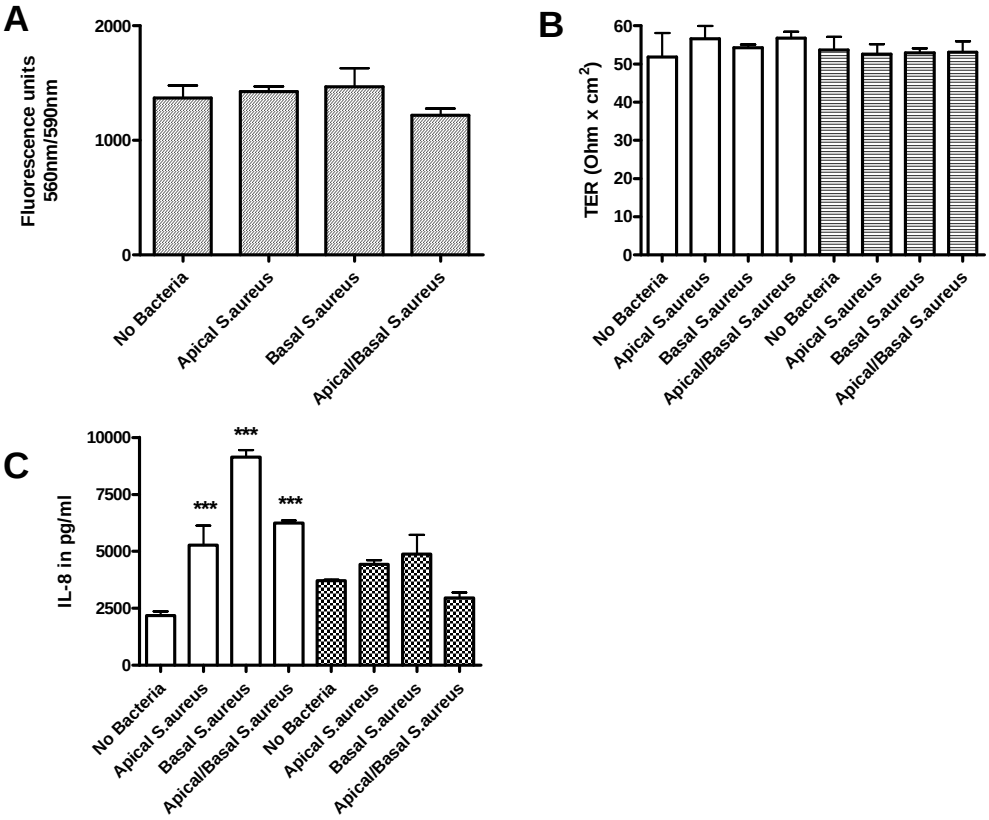

Figure 8.20 Cell viability (A), TER (B) and IL-8 release (C) of HPF-IB3-1 grown at ALI after exposure to HIA *S. aureus*
HPF-IB3-1 co-cultures were grown at ALI for 14 days before they were challenged with HIA *S. aureus*, which was applied either apically, basolaterally or from both sides at the same time. Control TWs were challenged with medium only. Cell viability of this co-culture was not significantly decreased after being challenged with HIA *S. aureus*. TER was found to be stable when comparing TER measured before (clear bars) and after 24 h exposure to HIA *S. aureus* (striped bars). IL-8 concentrations observed in apical supernatants (clear bars) showed a significant increase for all three challenges, and this was not observed in the basolateral medium (checked bars). Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1 co-cultures were exposed to HIA *S. aureus* for 24 h. HIA *S. aureus* were applied to the apical side of the co-culture, to the basal side or to both at the same time after 14 days of culture at ALI. Cell viability (figure 8.20 A) was not negatively influenced by these events (baseline fluorescence of control: 1370.54 ± 108.26 FU). TER (figure 8.20 B) was measured before (clear bars) and after (striped) the challenges and was observed to be unaffected by the exposure to HIA *S. aureus*. The measured TER of the control TWs was 51.88 ± 6.24 Ω cm^2 beforehand and 53.68 ± 3.46 Ω cm^2 afterwards. IL-8 secretion (figure 8.20 C) was assayed after the incubation of HPF-IB3-1 co-culture with HIA *S. aureus* and was significantly increased in the

apical supernatants after all three challenges compared to the control, which showed an IL-8 concentration of 2181.63 ± 181.49 pg/ml. In the apical supernatant (clear bars) after apical challenge, the concentration of IL-8 increased to 5279.72 ± 858.11 pg/ml, after the basally challenge it was 9137.52 ± 321.12 pg/ml and when this co-culture was challenged from both sides at the same time IL-8 concentration was measured at 6244.91 ± 109.76 pg/ml. Basolateral medium samples did not show this increase and were similar to the control, which had an IL-8 concentration of 3709.72 ± 55.66 pg/ml.

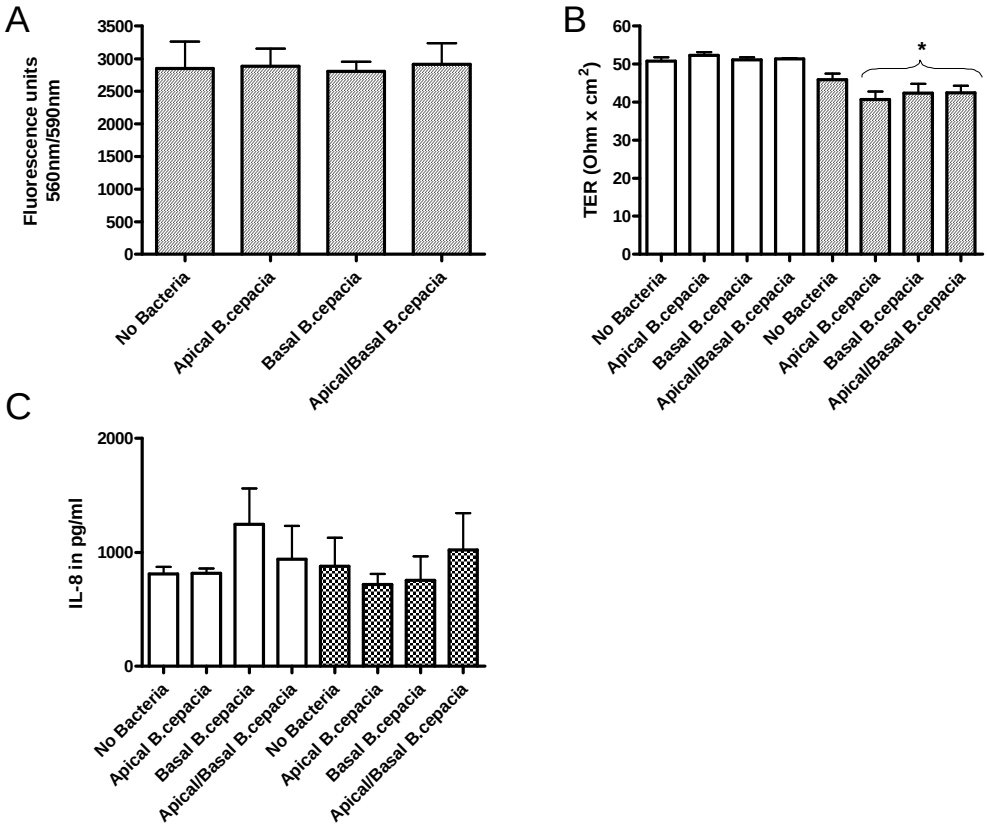

Figure 8.21 Cell viability (A), TER (B) and IL-8 release (C) after exposure to HIA *B. cepacia* of HPF-IB3-1 grown at ALI

HPF-IB3-1 co-cultures were grown at ALI for 14 days before they were challenged with HIA *B. cepacia*, which was applied either apically, basolaterally or from both sides simultaneously. HPF-IB3-1 on their own without bacteria were treated the same way as all other TWs and served as control. HPF-IB3-1's cell viability was not significantly affected by the exposure to HIA *B. cepacia*. TER was found to be significantly decreased after (striped bars) all three bacteria challenges, when compared to the corresponding TER from before (clear bars) IL-8 concentrations observed in apical supernatants (clear bars) and in basal media samples (checked bars) was not significantly changed. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1 co-cultures at ALI were exposed to HIA *B. cepacia* for 24 h. Bacteria were added either to the apical side, to the basal side or to both at the same time. Cell viability (figure 8.21 A) was not changed after this 24 h incubation for any of the challenges. Baseline fluorescence measured for control TWs was 2851.26 ± 410.85 FU. TER (figure 8.21 B) of this co-culture was monitored before (clear bars) and after (striped bars) this 24 h exposure to HIA *B. cepacia* and was found to be significantly decreased after all three challenges. Apically challenged co-cultures had a TER of 52.25 ± 0.94 Ω x cm^2 beforehand, which decreased to 40.70 ± 2.06 Ω x cm^2 after the challenge. Basolaterally exposure of HIA *B. cepacia* caused the TER to fall from 51.11 ± 0.73 Ω x cm^2 to 42.35 ± 2.49 Ω x cm^2. After the simultaneous challenge from both sides of co-cultures TER was found to be down to 42.5 ± 1.85 Ω x cm^2 compared to TER before challenge (51.37 ± 0.19 Ω x cm^2) Induction of IL-8 secretion (figure 8.21 C) could not be observed after this exposure to HIA *B. cepacia* and was 812.14 ± 61.23 pg/ml in the apical supernatant (clear bars) of the control and 879.69 ± 248.80 pg/ml in the basal medium (checked bars) of the control.

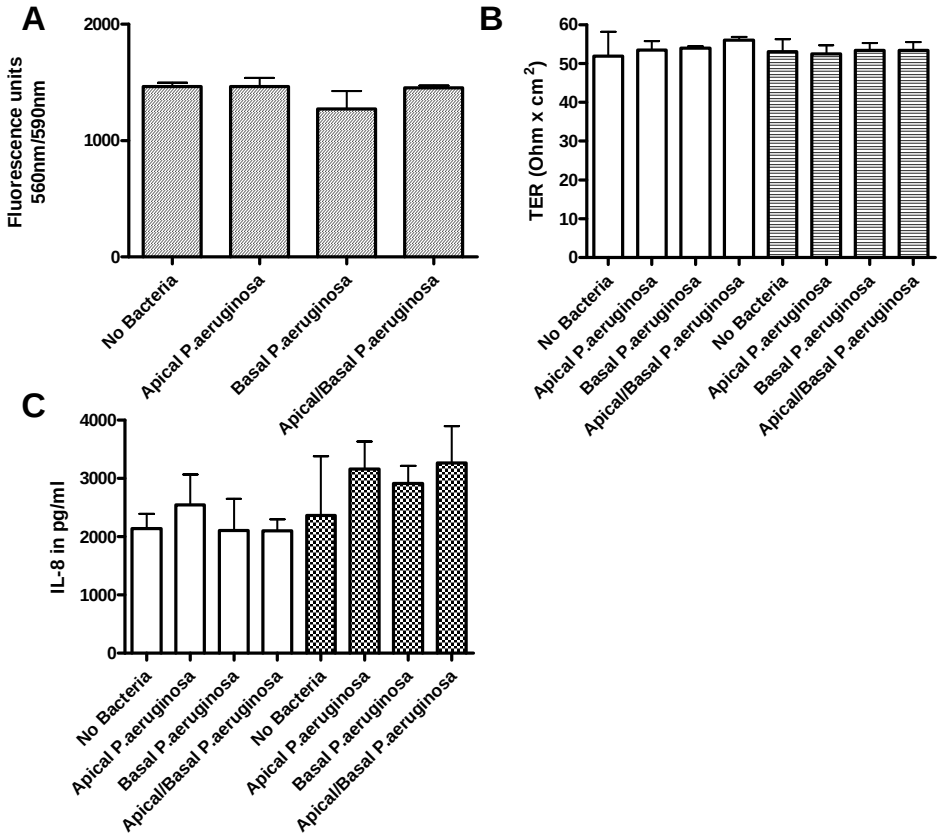

Figure 8.22 Cell viability (A), TER (B) and IL-8 release (C) after exposure to HIA *P. aeruginosa* of HPF-IB3-1 grown at ALI

HPF-IB3-1 co-culture were challenged with HIA *P. aeruginosa*, which was applied either apically, basolaterally or from both sides at the same time. HPF-IB3-1 on their own without bacteria were challenged with medium only and served as control. HPF-IB3-1' cell viability was not affected by this exposure to HIA *P. aeruginosa.* TER was found to be stable throughout the experiment for all three challenges after 24 h incubation (striped bars) when compared to their corresponding TER bar before the challenge. HPF-IB3-1 released equivalent levels of IL-8 into either the apical supernatants (clear bars) or into the basolateral medium compared to the control TWs. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-IB3-1's cell viability was assayed after 24 h exposure to HIA *P. aeruginosa*, which were applied either apically, basolaterally or from both sides at the same time and was found to be unaffected by this event. Cell viability (figure 8.22 A) was observed by CTB assay and the baseline fluorescence was 1462.93 ± 36.54 FU for the control co-cultures. TER (figure 8.22 B) was analysed before (clear bars) and after (striped bars) the exposure but was found to be constant throughout the experiment. Control co-cultures had a TER of 51.88 ± 6.24 Ω x cm^2 before the experiment and it was 53.06 ± 3.16 Ω x cm^2 after the challenge. Secretion of IL-8 (figure 8.22 C) was not induced by any of the challenges and was not significantly altered either in apical supernatants (clear bars) or in basal medium samples (checked bars). IL-8 concentration found in

control co-cultures was 2142.98 ± 251.64 pg/ml on the apical side and it was 2363.83 ± 1018.11 pg/ml on the basal side of the co-culture.

8.4.2.7 Exposure of HPF-Calu-3 co-culture to HIA bacteria

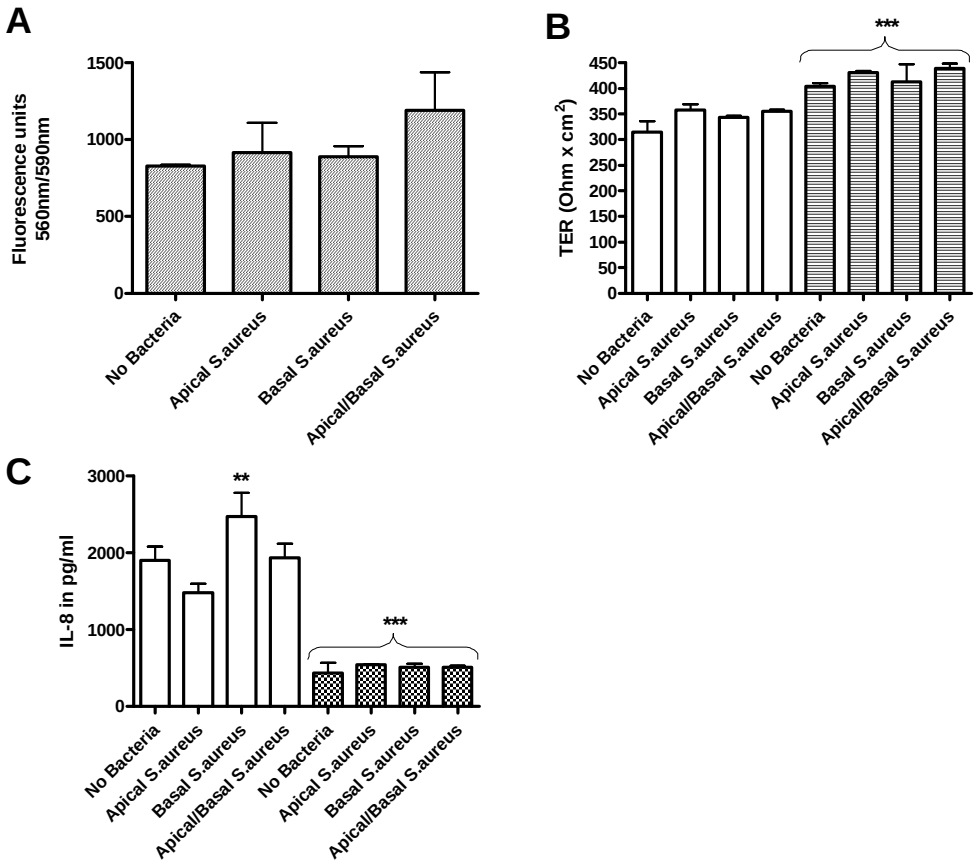

Figure 8.23 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 at ALI after exposure to HIA *S. aureus*
HPF-Calu-3 co-culture at ALI were challenged with HIA *S. aureus,* either apically, basolaterally or from both sides at the same time. HPF-Calu-3's cell viability was not significantly reduced by any of the 24 h exposures to HIA *S. aureus*. TER was measured before (clear bars) and after (striped bars) the challenges and was significantly increased for all TWs after this 24 h exposure to HIA *S. aureus*. IL-8 release was only increased significantly in apical supernatant (clear bars) following basolateral challenge. The basolateral IL-8 concentration (checked bars) was significantly lower in all TWs when compared to their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Co-cultures of HPF-Calu-3 were challenged with HIA *S. aureus* and the cell viability (figure 8.23 A) was investigated after a 24 h incubation with this bacterium. No significant changes were found compared to the control, which showed a baseline fluorescence of 829.37 ± 8.28 FU. The co-cultures TER (figure 8.23 B) was monitored before (clear bars) and after (striped bars) the

challenges, where *S. aureus* was applied either apically, basolaterally or simultaneously from both sides. The control TWs measured a mean TER of 314.08 ± 21.73 Ω x cm^2 before the exposure to this bacterium and 404.36 ± 6.14 Ω x cm^2 afterwards, which was significantly higher. All the other TERs were significantly increased after the challenges. Apically challenged TWs increased from 357.57 ± 11.21 Ω x cm^2 beforehand to 430.97 ± 2.99 Ω x cm^2 after the challenge. When exposed to HIA *S. aureus* from the basolateral side the TER was 413.09 ± 33.81 Ω x cm^2 after the exposure compared to 343.68 ± 2.79 Ω x cm^2 beforehand. TWs that were exposed apically and basolaterally to HIA *S. aureus* showed a significant increase in TER as well, which was 355.41 ± 3.03 Ω x cm^2 before the challenge and 438.75 ± 9.52 Ω x cm^2 after the challenge. IL-8 concentration (figure 8.23 C) in the apical supernatants (clear bars) was measured and only found to be significantly increased after basolateral challenge of co-cultures of HPF-Calu-3. The IL-8 concentration measured in this case was 2470.4 ± 314.28 pg/ml compared to 1902.38 ± 178.01 pg/ml found in the control. Basolateral medium samples (checked bars) were analysed alongside and no significant increases could be observed for these compared to the basal control medium. However, for this co-culture basolateral medium samples all together showed a significantly lower concentration of IL-8 compared to their corresponding apical sample.

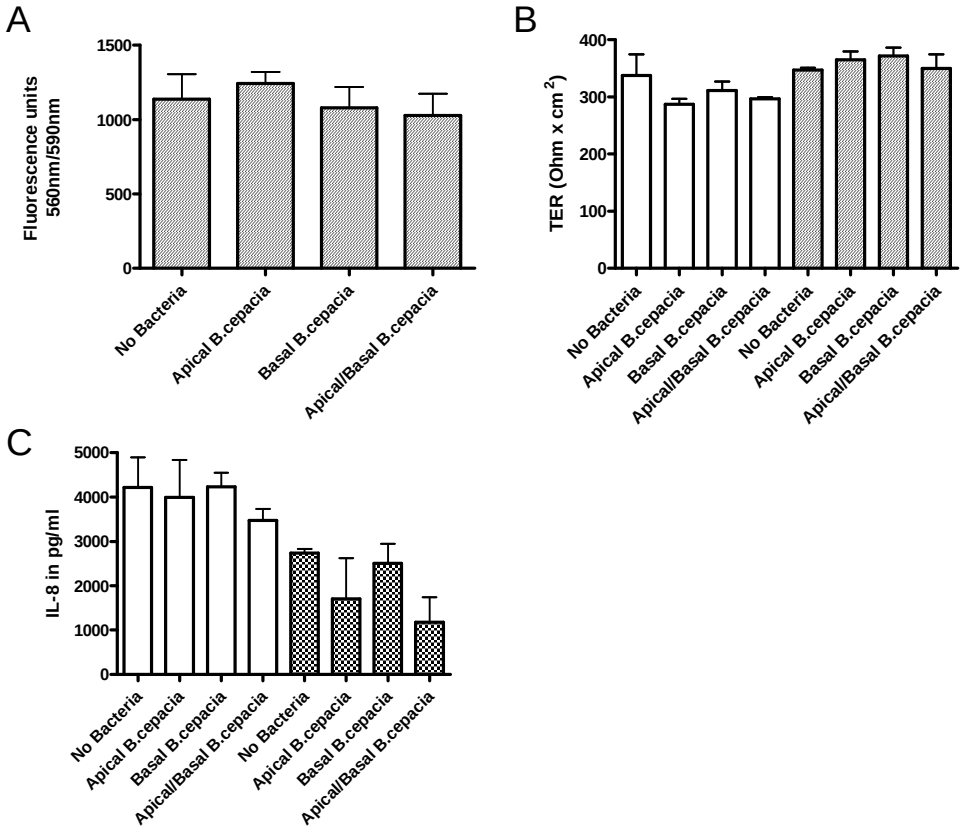

Figure 8.24 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to HIA *B. cepacia*

HPF-Calu-3 co-cultures at ALI were, either apically, basolaterally or from both sides at the same time, challenged with HIA *B. cepacia*. Cell viability of this co-culture was not significantly decreased when challenged with HIA *B. cepacia*. The TER was measured before (clear bars) and after (striped bars) the challenges and was found to be stable throughout the experiment. IL-8 concentrations in the apical supernatants (clear bars) were not significantly increased after 24 h incubation with HIA *B. cepacia*. The basolateral sample IL-8 content (checked bars) was found to be significantly lower than their corresponding apical supernatant. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

Co-cultures of HPF-Calu-3 grown at ALI were exposed to HIA *B. cepacia* for 24 h. The bacteria were applied either apically, basolaterally or from both sides at the same time, which did not influence the cell viability (figure 8.24 A), which was measured straight after exposure. Baseline fluorescence that was observed for control was 1137.85 ± 166.70 FU. Furthermore TER (figure 8.24 B) was analysed before (clear bars) and after (striped bars) these exposures and was not changed either after 24 h incubation with HIA *B. cepacia*. The control co-cultures, which were exposed to SF-medium only, had a TER of 337.70 ± 36.65 Ω x cm^2 beforehand and 347.05 ± 4.22 Ω x cm^2 after the challenges. IL-8 release (figure 8.24 C) by this co-culture was not enhanced by

the exposure to HIA *B. cepacia*, neither in the apical supernatants (clear bars) nor in the basaolateral medium samples (checked bars).

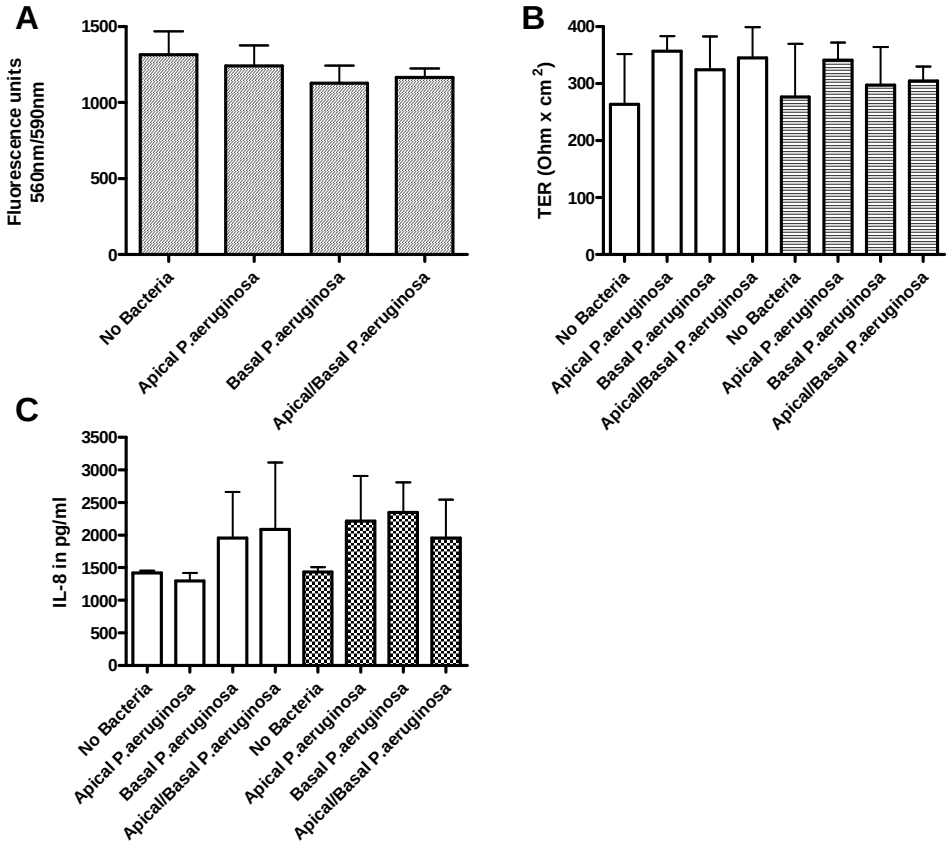

Figure 8.25 Cell viability (A), TER (B) and IL-8 release (C) of HPF-Calu-3 co-culture after exposure to HIA *P. aeruginosa*
HPF-Calu-3 co-cultures at ALI were challenged with HIA *P. aeruginosa,* either apically, basolaterally or both at the same time after 14 days of culture. None of the measured parameters were changed significantly. Cell viability (A) was not affected by this challenge, TER (B) was not changed after 24 h incubation with HIA *P. aeruginosa* and the IL-8 concentrations (C) did not show any significant increases compared to the control. Data are presented as mean ± SD of 3 individual experiments (3TWs each, n=3).

HPF-Calu-3 co-cultures were exposed to HIA *P. aeruginosa* for 24 h at ALI. Bacteria were applied either apically, basolaterally or simultaneously on both sides, which did not have any affect on cell viability (figure 8.25 A). The control co-cultures, which were exposed to SF-growth medium only showed a baseline fluorescence of 1316.89 ±151.52 FU. TER (figure 8.25 B) was assayed before (clear bars) and after (striped bars) the challenges and was not seen to be different after these exposures to HIA *P. aeruginosa*. Co-cultures that served as control had a TER of 264.11 ±

87.92 Ω x cm^2 beforehand and 276.47 ± 93.61 Ω x cm^2 afterwards. IL-8 concentrations (figure 8.25 C) of all apical supernatants (clear bars) as well as of all basal medium samples (checked) were analysed and no significant differences were observed compared to the control, which had 1420.59 ± 29.49 pg/ml IL-8 in the apical supernatant and 1437.87 ± 75.11 pg/ml in the basal medium.

8.5 Discussion

After the established mono- and co-culture models have been tested for their ability to respond to LPS from various CF relevant pathogens and for their ability to respond to CF relevant live bacteria, in this chapter they have been exposed to heat-inactivated bacteria. Heat-inactivation of bacteria is one of the most widely used methods for destruction of pathogenic bacteria, which are then not able to produce and release, for example, exotoxins. In this chapter, therefore the induction of an inflammatory response in the culture models by heat-stable cell wall components of bacteria, such as LPS in gram-negative and LTA in gram-positive bacteria were analysed. This method of stimulating the models with heat-inactivated bacteria was carried out to see the differences in response compared to live bacteria, when all exoproducts and secreted virulence factors are not present.

Here the results of challenging all the different models with each different heat-inactivated pathogen will be discussed together as there were not many significant changes after exposure of mono- or co-cultures to HIA bacteria.

As these bacterial suspensions were washed several times after heat-inactivation and before determining the OD of bacterial suspensions it is most likely that all exoproducts and secreted virulence factors were eliminated from this experiment. No free LPS or LTA (or at least very low levels) were present in these experiments. Comparisons of these data with the models' response to intact, live bacteria and concentrated, isolated LPS might help to elucidate further, the virulence of intact bacteria compared to heat-inactivated bacteria and shed some light on the ability of heat-stable cell wall components to induce an inflammatory response.

After challenges with any of the HIA bacteria, no changes in cell viability for any of the models were observed. This shows that none of the heat-stable cell wall components are able to disrupt the epithelial cell membrane and no cytotoxic effect was observed. This indicates that only intact bacteria have the ability to decrease cell viability, as discussed in chapter 7. In that chapter it was observed that after *S. aureus* challenges the cell viability in epithelial cell mono-cultures at ALI and all three co-cultures was significantly decreased, which was not observed here or for LPS. This was similar for live *P. aeruginosa* as there was a significant decrease in cell viability of C38 and IB3-1 mono-cultures at ALI and also in the co-cultures of these two cell lines. These effects could not be observed here for HIA *P. aeruginosa*. The only similarity in terms of decreased cell viability was seen for IB3-1 mono-cultures at ALI as *P. aeruginosa* 50 DR LPS decreased the cell viability of this mono-culture after the basal and apical/basal challenge but the cell viability was decreased to a greater extent after challenges with live bacteria, as here the decrease was observed after all three challenges with *P. aeruginosa* 50DR. No decrease was observed after

HIA *P. aeruginosa* stimulation. This indicates that there is a difference of the cytotoxicity of purified LPS and the HIA bacteria, which still contain LPS in the outer membrane. This is possibly down to Lipid A being mostly membrane bound after the heat-inactivation, whereas this part of LPS is free in purified LPS. It has been reported before that Lipid A is largely responsible for the cytotoxicity of LPS (Rietschel *et al*., 1994).

8.5.1 HIA *B. cepacia* is able to compromise cell layer integrity

For the cell layer integrity it was surprising to observe a decrease in TER after the challenges with HIA *B. cepacia*. This decrease was seen for IB3-1 mono-culture at ALI, for HPF-C38 at ALI and for HPF-IB3-1 at ALI. These findings suggest that HIA *B. cepacia* was still able to compromise the epithelial cell layer integrity, even after HIA. The mechanisms behind this virulent trait are unknown but different results were observed after the challenge using live bacteria. The TER has not been found to be compromised after this 24 h challenge even though in HPF-C38, HPF-IB3-1 and HPF-Calu-3 there was some degree of decreased cell viability. Again it becomes clear here that TER is not necessarily a good indicator of cytotoxicity. In addition TER was only measured after 24 h and only shows what was different at this time point compared to the situation before bacterial exposures. Not much research has been done up until now that looks at exact mechanisms of the virulence factors on TER over time but there is one report that shows time-courses of measured TER across human gingival keratinocytes with and without infection using *Porphyromonas gingivalis* (*P. gingivalis*). In this report it is shown that whole *P. gingivalis* is only able to modulate the TER upon a certain bacterial concentration used to infect the cells with. Above that concentration TER has been shown to increase first before it drops down to almost zero after 24 h following apical challenges. Different results were found for basal and simultaneous apical/basal exposure to this bacterium as the period of increase was not observed at all and instead the TER started dropping after only two hours of infection and after 8 h reached nearly zero following the basolateral challenge and following the apical/basal challenge TER almost reached zero after only 6 h (Groeger *et al*., 2010). These findings indicate that the cell layer integrity can be dynamically changed over a short period of 24 h and points out that a snapshot of one time point will only tell a bit of the story and that it is not possibly to conclude from the end result, what might have been going on in between. To see whether the drop in TER identified after 24 h following the HIA challenges with *B. cepacia* might rapidly recover, has to be investigated further. As no decrease in cell viability has been observed here the epithelial cells should be able to re-establish cell layer integrity.

Another point to note here, is that Calu-3 mono- and co-cultures do not show a decrease in TER following the HIA challenge with *B. cepacia*. However, following the challenge with live/intact *B.*

cepacia the TER was compromised in the mono-culture after the simultaneous exposure to *B. cepacia* and was also compromised in the co-culture of HPF-Calu-3 but here following all three challenges. This suggests that there is a different dynamic interaction between the bacterium and different cell lines, which could lead to different level of potential for the bacterium to be able to disrupt cell layer integrity. The exact mechanisms behind these interactions are not fully elucidated yet.

However for all these results presented here it needs to be kept in mind that the epithelial cell layer was not compromised completely and equilibration of the IL-8 concentration was not observed. TER measurements are not a direct indicator of cytotoxicity. No decrease in cell viability was observed for the HIA challenges but a decrease in TER. HIA *B. cepacia* binding to epithelial cells and the possible modulation of tight junctions has not been investigated here and it is impossible to make a statement about the ability of HIA bacteria to compromise the cell layer integrity, at least to a certain degree.

8.5.2 HIA *S. aureus* stimulates IL-8 secretion

IL-8 secretions after challenging the mono- and co-culture models with HIA bacteria were only observed in a few cases. Out of the submerged mono-cultures only Calu-3 responded to *S. aureus* and to *P. aeruginosa* but they did not following the challenges when cultured at ALI. HIA *S. aureus* was also able to induce an inflammatory response in terms of IL-8 secretion in HPF-C38 and HPF-IB3-1, with the latter responding to all three challenges, whereas HPF-C38 only responded after the simultaneous apical/basal challenge with HIA *S. aureus* and this underlines once more the higher IL-8 responsiveness of HPF-IB3-1 to this stimulus under these conditions. Furthermore, HPF-Calu-3 responded to the basal challenge with *S. aureus*. This shows that following the challenges with HIA *S. aureus* only the co-culture models respond with secretion of IL-8 and no changes in cell viability or TER were observed after these challenges. In comparison to the challenges using live *S. aureus* it becomes clear that the results concerning IL-8 secretion are very similar as HPF-C38, HPF-IB3-1 and HPF-Calu-3 respond but none of the mono-cultures apart from HPF. However, live *S. aureus* is more virulent to these cultures, when in its intact form, as *S. aureus* was able to decrease the cell viability in all mono- and co-cultures, apart from HPF mono-culture, which did not show a decrease in cell viability. Additionally in the co-cultures *S. aureus* was also able to disrupt the epithelial cell layer and decrease the TER measured afterwards, which is possibly down to other virulence factors rather than heat-stable cell wall components and to the dynamic interactions of the pathogen and the cell cultures. Interestingly following both different challenges (live, HIA) of the co-culture models the IL-8 secretion seems to be mostly directional to the apical side of the culture.

It has been reported before that the gene expression profile of keratinocytes, for example, is different in response to live or HIA *S. aureus* and it was pointed out there that the different gene expression profiles highlight the variability and complexity of analysing host responses to inactivated bacteria even when the pathogen repertoire is reduced in killed bacteria (Menzies and Kenoyer, 2005).

8.5.3 Submerged Calu-3 mono-cultures

Calu-3 mono-cultures were not stimulated by HIA gram-negative bacteria, when cultured at ALI. Only under submerged conditions did these cells respond to HIA *P. aeruginosa* and as discussed above an induction of IL-8 secretion after *S. aureus* exposure under submerged conditions was seen.

These results indicate that Calu-3 cells recognise pathogens differently under these different culture conditions. Whether this is down to direct binding to PRRs or to different adherence mechanisms has not been investigated here. This tremendous difference in response to pathogens comparing submerged and ALI cultures, points out once more that culture conditions and epithelial cell differentiation make a difference to the cellular response.

In contrast to these findings, another group has reported that using HIA *P. aeruginosa* to stimulate the human bronchial non-CF epithelial cell line 16HBE14o-, the CF cell line CFBE41o and the CFTR corrected CFBE 41o- did lead to an increase in IL-8 concentration when grown at ALI. For all of these cell lines a significant increase of IL-8 was observed following the bacterial challenge (John *et al*., 2010). This is different to what was observed in these experiments presented here as no increase of IL-8 was observed following the challenges with HIA *P. aeruginosa* on ALI mono- and co-cultures. This underlines that different cell lines from different backgrounds respond differently to different stimuli.

Heat inactivation of bacteria reduces the immunomodularity potency and virulence of these bacteria, which is not surprising when the effect of heat on bacteria is taken into account. Bacteria are dead and cannot actively multiply and furthermore they cannot produce anymore virulence factors, which will be mostly destroyed by the heat-inactivation. Apart from LPS and LTA, which are heat stable to a certain degree, most other bacterial components such as RNA, DNA, proteins, enzymes and membranes are heat sensitive (Lee and Kaletunc, 2002). As bacterial suspensions were washed twice before the OD was adjusted for experiments all

secreted virulence factors, including exotoxins were eliminated and were not the cause of induced IL-8 secretion after *S. aureus* challenged cultures.

Heat-inactivated bacterial whole-cell vaccines have been widely used for infectious diseases, which are necessary because of arising antibiotic resistances by many bacteria and a major advantage is that a broad collection of antigens can be presented to the immune cells at one point, plus these cause minimal side effects because the virulence potential is decreased (Pace *et al*., 1998).

9 Chapter 9 Discussion

9.1 Discussion

The overall aim of this project was to develop co-culture models of normal and CF human airways *in vitro*. These co-culture models were established using HPF and the epithelial cell lines C38, IB3-1 and Calu-3. In the first part of this project it was all about characterising these models but not only the established co-culture models were characterised also the mono-cultures of each cell type was analysed. Mono-cultures were employed to all experiments throughout this work to be able to identify cell type specific characteristics, which could then be used for support in analysing the co-culture data. Throughout the first three chapters several essential basics were determined to be able to establish a co-culture model. Human placental collagen type IV was determined to be a supportive growth substrate for these mono-and co-culture models and further it was observed that co-cultures could be grown in the epithelial medium of the epithelial cell line used, whereas mono-cultures were all kept in their cell-type specific medium. An anti-vimentin antibody and an anti-CK8 antibody were identified as cell type specific and these be used for identifying HPF and epithelial cells, respectively in paraffin embedded cross sections of the co-culture models. Using the anti-vimentin antibody on paraffin embedded cross-sections it was shown that no fibroblastic overgrowth occurred in the co-cultures and fibroblasts were located subepithelial. Further characterisation was achieved by analysing epithelial cell layer integrity, which was presented by barrier formation and these were measured using TER measurements. Apical expression of the tight junction protein ZO-1 was shown for all epithelial cells and staining patterns indicated an intercellular location of this protein- characteristic of the peripheral nature of the tight junctions in native airways epithelia. Directed, apical MUC5AC secretion was observed from epithelial cells at ALI- in mono- and co-cultures. Preliminary analysis also revealed the presence of proteolytic activity (in the form of activated MMP), however, this was only observed for apical secretions from the CF model.

Following these characterisations in the second part of this project bacteria as well as bacterial related stimuli (LPS and HIA bacteria) were used to investigate pro-inflammatory responses of these mono- and co-cultures. In addition, the effects of these stimuli on cell viability and epithelial integrity were measured.

LPS stimulation of HPF mono-cultures revealed that fibroblasts under submerged conditions, in 24 well plates, and fibroblasts grown under submerged conditions on TWs respond differently to LPS, which might be a result of the formation of a multi-cell layer of HPF on TWs compared to mono-layers when grown in 24-well plates. Different results were also observed for epithelial

cells, when comparing data from submerged cultures with mono-cultures grown at ALI. Furthermore the results obtained from mono-culture challenges were mostly different to the results from co-cultures. Similar differences were observed when analysing the mono- and co-culture models results after stimulation with live or HIA *S. aureus*, *B. cepacia* or *P. aeruginosa*, indicating a general lack of pathogen-specific responses.

The main points to summarize from the data presented in chapters 7 and 8 were that HPF clearly responded to bacterial challenges and could therefore play an important role in the pathogenesis of CF airways disease. Furthermore this study has shown evidence of a hyper-inflammatory response in the CF model; thus, IB3-1 mono-cultures respond to challenge with significantly more IL-8 secretion compared to C38, which was also mostly the case in IB3-1-HPF co-cultures. Interestingly it was also observed for cell layer integrity that, where cultures were affected by live bacterial exposure, then mono- or co-cultures of IB3-1 were usually more susceptible. For challenges with HIA bacteria it was found that the virulence of bacteria was decreased tremendously and therefore the effect of the bacteria on cell viability was reduced accordingly. Most interestingly, it becomes clear from comparing data from live and HIA challenges that mono-cultures react completely differently to co-cultures challenged with the same pathogen. These data therefore generate questions about the exact role HPF play in pro-inflammatory responses in the airways, as well as what the exact mechanisms are of intercellular communication and which secreted chemokines, cytokines and growth factors may play a role in these responses.

9.1.1 Cell culture models of diseased airways

In general, *in vitro* cell culture models have been developed to mimic different tissues and microenvironments and they are simplified versions of certain organs and tissues, such as the models presented here, which have been designed to mimic the bronchial airways of normal and CF human airways. Three dimensional cell culture models have gained in popularity over the last decade and have been used in pathophysiological and biomedical research to investigate normal and diseased tissues (Parker *et al*., 2010), to study drug delivery (Foster *et al*., 2000), cell responses to different stimuli (Becker *et al*., 2004) or to observe signalling pathways (Nilsson *et al*., 2010), just to name a few. However, these *in vitro* cultures are only mono-cultures employing one cell type, which does only mimic a small area of the tissue, such as epithelial cells

of the airways. *In vivo* there are many other important cell types present, such as the subepithelial fibroblasts and other immune cells, such as neutrophils and macrophages.

A few studies employing different variations of *in vitro* and *in vivo* co-culture models for airway related research have been published, not only for CF (Wiszniewski *et al*., 2006) but also for COPD and asthma (Choe *et al*., 2003), for example. However, in the co-culture model published by Wiszniewski, for example, the subepithelial fibroblasts are grown on the undersurface of the TW and are postmitotic, which does not mimic the *in vivo* situation. *In vivo*, there are controlling mechanisms for cell and tissue homeostasis, which need to be investigated to find out whether these are altered in disease and therefore determine if they might play a role in pathological settings.

9.1.2 Selecting an appropriate epithelial cell to model the airways

There are many immortalised epithelial cell lines available for airways research and all of these have advantages and disadvantages. In this study, the Calu-3 cell line was evaluated since this cell type is very well characterised in drug delivery studies, expresses CFTR and develops measureable TER (Shen *et al*., 1994, Grainger *et al*., 2006). However, Calu-3 were found to be very different compared to the other two cell lines for all the characteristics analysed.

The expression of cell type specific markers were determined with the aim of using antibodies directed towards these markers for cell type identification in the co-culture models (HPF and epithelial cells). As discussed in chapter 4, cytokeratins (CK) are epithelial specific intermediate filaments that allow researchers not only to discriminate between tissue specific epithelial cells but also allow to distinguish the stage of differentiation with respect to their CK expression (Purkis *et al*., 1990). For this reason antibodies have been developed against these proteins and the two antibodies used in this project were raised against CK5 and CK8. CK5 is a marker for basal epithelial cells, whereas CK8 is strongly expressed in differentiated simple epithelial cells and has also been found in the respiratory tract, where it is mainly found in the lumen lining cells (Moll *et al*., 2008, Purkis *et al*., 1990). The CK staining pattern of Calu-3 that was observed here showed strong positive staining for both CKs, whereas IB3-1 and C38 were negative for CK5 under submerged conditions and only showed very weak staining at ALI. In chapter 4 it was discussed in detail that several things can influence the expression profile of epithelial cells, including cancer. It has been summarized by Moll *et al*. (2008) that typical expression profiles appear in different types of carcinomas and according to this there should be no CK5 expression

or very low level CK5 in lung adenocarcinomas (from which Calu-3 were derived originally). However, when Calu-3 were initially characterised they were shown to express CK5, observed as patchy staining (Daniel and Burnett, 1991) indicating that these epithelial cells do not behave typically like cells from an adenocarcinoma but neither do they behave like normal epithelial cells of the airways, which will be discussed further.

Another epithelial cell feature important for models in airway research is to establish a reasonable barrier by developing a tight epithelium as adjacent cells tightly connect through tight junctions, which regulate ion flow across the apical membrane of epithelial cells. The barrier formation was monitored here by using a voltohmmeter with chopstick electrodes to measure electrical resistance across the cell layer (TER). The maximal TER (figure 5.3) of Calu-3 in mono-culture and co-culture was measured at 399.56 ± 37.99 Ω x cm^2 and 291.96 ± 14.91 Ω x cm^2, respectively. It is important to note that HPF-Calu-3 co-cultures did not reach the same value as the mono-cultures and were still significantly lower after 14 days of culture at ALI. These substantial differences in TER suggest that either the Calu-3 mono-cultures develop artificially high values that are normalised in the presence of fibroblasts, or that the fibroblasts somehow modify the barrier characteristics of Calu-3. The reasons remain to be determined, but cast further doubt on the usefulness of Calu-3 in co-cultures. This difference in TER on day 14 of ALI culture of mono- and co-culture was not observed for the other two epithelial cell lines. Additionally the TER of Calu-3 mono-cultures were seven times higher than the TER of C38 or IB3-1 mono-cultures, suggesting functional differences between Calu and other epithelial cells. Similarly the TER of HPF-Calu-3 co-cultures were more than 5 times higher than HPF-C38 or HPF-IB3-1 co-cultures. These data all suggest that Calu-3, whilst remaining a good choice for barrier studies where a high TER is a necessary feature, cannot be used in direct comparison with other epithelial cell models, such as IB3-1.

TER cannot be correlated directly to ZO-1 protein expression (Claude and Goodenough, 1973) but the staining pattern of ZO-1 and strength of staining usually is in accordance with this. For Calu-3 there is a wide variability reported for TER throughout the literature. There are values ranging from around 300 Ω x cm^2 (Grainger *et al*., 2006, Zhang *et al*., 2001b), which is similar to what was observed here, to 1000 Ω x cm^2 (Mathia *et al*., 2002). The fact that the TER of Calu-3 was so much higher compared to C38 and IB3-1 pointed out that Calu-3 cannot be a direct control in terms of non-CF, for IB3-1 (CF) as this would eliminate one of the required CF model features, which is a higher TER compared to the non-CF control. Therefore only C38 was used as

a direct control for IB3-1. It was shown in chapter five that HPF-IB3-1 had a significantly higher TER over the 14 day period compared to HPF-C38 and shows that this disease feature was presented by these models, as it has also shown for CFBE410- (CF) and CFBE41o-pCep4 (non-CF) mono-cultures (Nilsson *et al*., 2010).

The lack of relevance of the Calu-3 as a non-CF model was also true for some other features analysed here. Apical secretions of each mono-culture and co-culture were investigated for the presence of MUC5AC – the main mucin species found in the airways. All epithelial cell lines secreted MUC5AC into the apical secretions following culture at ALI, but Calu-3 constitutively expressed and secreted MUC5AC, even in submerged culture (figure 5.7). Dot blot analysis of Calu-3 mono-culture secretions (figure 5.7, lane C) revealed that MUC5AC was already strongly expressed before ALI was established and this was also true for apical secretions of HPF-Calu-3 (figure 5.7, lane F). This constitutive expression of mucin by Calu-3 cells has been reported before, where it was also found that mucin secretion was not different for cells cultured on glass-cover slips or on TWs (Kreda *et al*., 2007). In contrast, C38 and IB3-1 secreted MUC5AC only after ALI was established showing another important difference between these cells and Calu-3 and again suggesting that direct comparisons between Calu-3 as a non-CF model and IB3-1 as a CF model are flawed. Dehydrated mucus and inefficient mucus clearance is one of the main problems observed in CF lung disease (Boucher, 2007) and therefore *in vitro* models for CF research should provide this feature for investigation but again there is such difference compared to the other cell lines that Calu-3 cannot serve as direct non-CF control for IB3-1.

Even though Calu-3 have been shown before to express high levels of CFTR (Shen *et al*., 1994), which is one requirement for a good non-CF control, they have been shown to represent a mix of epithelial cell phenotypes with an important goblet cell component (Kreda *et al*., 2007) and throughout this work it has been shown that Calu-3 are very different in phenotypic characteristics as well as cell behaviour compared to C38 and IB3-1. The differences were also apparent when analysing inflammatory responses of all three epithelial cell lines to LPS. The first big difference is that Calu-3 respond to all three types of LPS, whereas C38 only responds to *B. cepacia* and IB3-1 does not respond at all. Furthermore C38 only responded to 100 ng/ml and 1000 ng/ml of *B. cepacia* LPS (figure 6.4), whereas Calu-3 respond to all concentrations used apart from 0.1 ng/ml (figure 6.10). The responses in terms of IL-8 secretion were more than 80 times higher in Calu-3 supernatant compared to C38 at the LPS concentration of 100 ng/ml and 1000 ng/ml and the baseline IL-8 was also about 60 times higher comparing Calu-3 to C38. For mono-cultures at ALI it was observed that Calu-3 is the only cell line responding at all and again

showing a baseline IL-8 secretion of more than 3500 pg/ml, whereas control levels of IL-8 for the other cell lines was below 200 pg/ml. These huge differences in IL-8 secretions would make direct comparisons impossible and prevent validation of IB3-1 as a hyperinflammatory model that mimics the CF phenotype.

9.1.3 Novel *in vitro* co-culture models to investigate CF bronchial airways – is there a need for these?

Initially, this work concentrated on the characterization of these mono- and co-culture models, whereas the second part of the project focussed on assessing the functionality of the developed models, which was concentrated on inflammatory responses to LPS, intact bacteria as well as HIA bacteria. Altogether novel, valuable and functional **co-culture** models were developed, which represent a more complex model system, compared to the mono-culture systems available, as these include subepithelial fibroblasts. These novel models will enable the investigation of intercellular communications between epithelial cells and fibroblasts and as these co-culture models were established as non-CF and CF models these can help to elucidate differences of cell communication and behaviour, which could possibly be related to the major difference between C38 and IB3-1, which is the expression of CFTR.

For *in vitro* cell culture models of airway epithelial cells it has been reported that cells grown on a growth substrate, which usually are components of the ECM found *in vivo*, such as collagens, fibronectin and laminin, show enhanced cell adherence (Wiesel *et al*., 1983). Furthermore it has long been recognised that *in vivo* cell-ECM interactions are important for normal growth and differentiation (Grobstein, 1967) as well as for some biological functions, such as adhesion (Hinenoya *et al*., 2008), proliferation (Robinson and Wu, 1993), migration and differentiation (Vlodavsky *et al*., 1980, Hinenoya *et al*., 2008). Plus two of the surface membrane receptors, which belong to the integrins, have been shown to be collagen receptors and are constitutively expressed in the human adult lung *in vivo* as well as in cultured primary human airway epithelial cells *in vitro* (Wang *et al.*, 1996). Collagen IV is a major component of the BM (Timpl, 1989) and is known to underlie the epithelium *in vivo* (Sage*,* 1982). In addition Fiedler *et al*. (1991) has shown that epithelial cells grown on collagen retain the ability to produce secretory component, which is a receptor for dimeric IgA and this complex is eventually released to the lumen of the airways to prevent microorganism adherence to the mucosal surface and is therefore an important part if the immune defence in the airways (Fiedler *et al*., 1991).

Here it was also shown that cell adherence to collagen-IV coated plastic ware was enhanced compared to cell adherence to plain plastic surfaces (chapter 3). Collagen IV is only one component of ECM but one reason for the addition of fibroblasts to the models was that fibroblasts are the main producers of ECM components (Mutsaers *et al*., 1997). Adding fibroblasts to the model would therefore provide a source for naturally produced BM components, which would mimic the *in vivo* BM more closely, helping to make these models more relevant. It has been shown previously that primary fibroblasts lay down ECM components and furthermore that bronchial epithelial cells grow better on ECM produced by fibroblasts compared to standard collagen coated wells (Skibinski *et al*., 2007). The adhesion of epithelial cells to fibroblasts was not evaluated during this study, but the development of an epithelial cell-dependent TER in co-cultures indicates successful growth of an epithelial cell layer on the fibroblasts and immunohistochemistry staining of paraffin-embedded sections confirmed the presence and relevant location of the two distinct cell populations, with fibroblasts remaining in close contact with the TW membrane.

Furthermore for the establishment of the co-culture models the cell culture growth medium is important, as this delivers essential nutrition, such as growths factors and hormones to support proliferation and differentiation, for example (Moghal and Neel, 1998). HPF were shown to proliferate normally, when grown in epithelial cell growth medium, which were therefore used to establish co-culture models. Having successfully determined general but essential growth conditions the next aim was to find antibodies against cell type specific markers for distinguishing between fibroblasts and epithelial cells. As the fibroblasts were seeded subepithelial without an actual physical barrier in between the cell types, this was essential. Anti-CK8 and anti-vimentin were the antibodies with highest specificity and would therefore be used in future experiments to localise cell types in cross sections of these co-culture models. Here it was shown for the first time that actively proliferating fibroblasts grown subepithelial in co-culture with epithelial cells do not overgrow the epithelial cell layer, which in the presence of fibroblasts established a pseudo-stratified like appearance (figure 4.12). It has been also been mentioned in the work presented by Skibinski *et al*. (2007) that human bronchial fibroblasts, if not treated to be postmitotic (mitomycin C), will expand by three fold in ten to twelve days (Skibinski *et al*., 2007). However, this was not observed throughout this work and highlights once more that there is important communication going on between epithelial cells and fibroblasts, which seem to regulate cell growth.

9.1.3.1 Epithelial-fibroblast interaction promotes normal epithelial cell differentiation

Mono-cultures of HPF and of each epithelial cell line were analysed alongside the co-cultures in each experiment because as already mentioned above it was important to analyse mono-culture features in turn to be able to verify cell type specific features and allocate them to one of the two cell types present in the co-culture if possible, such as shown for TER.

In this study valid and functional co-cultures models are presented, which show that fibroblasts support epithelial differentiation. Even though these co-culture models are in their infancies they were proven to sustain essential characteristics of the airways *in vivo*.

One of these characteristics is the formation of a tight epithelial cell barrier, which was shown to be established in mono-cultures. Further characterisation revealed that all co-culture models were also able to establish a tight barrier and considering that HPF only form a very weak physical barrier with an average TER of 8.85 ± 0.89 Ω x cm^2 over a period of 14 days it is clear that this barrier formation is of epithelial origin (figure 5.1-5.3). Positive ZO-1 protein staining of the epithelial cells in mono- and co-culture (figure 5.5) further supported the identification of a tight epithelial cell layer.

Another essential feature for models of CF airways *in vitro,* is mucus secretion. Mucus hypersecretion is one of the hallmarks of CF and is typically associated with airway inflammation as seen in CF. All the co-culture models presented here were observed to secrete MUC5AC into the apical secretions of each model over a period of at least 14 days (figure 5.6 and 5.7). Mucin secretion was related to differentiation of the airways, but this project did not to determine whether the IB3-1 cells secreted greater levels of mucin, as expected in CF, or if the mucins displayed the typical CF-dependent-changes in glycosylation/sulphation (Xia *et al*., 2005). For further experiments to analyse mucin quantity, for example, C38 and IB3-1 will be compared to validate whether IB3-1 hypersecrete mucus as it is seen in CF airways. Dehydrated mucus and inefficient mucus clearance is one of the main problems observed in CF lung disease (Boucher, 2007) and therefore *in vitro* models for CF research should ideally provide this feature for investigation.

Additionally, this work has shown that HPF promote expression of microvilli and immature cilia in the epithelial cells (figure 4.14-4.17). This confirms observations from a previous similar study where fibroblasts were located on the undersurface of TWs and it was suggested that the promoting effect observed, was either an increase in the number of ciliated cells or prolonged survival of ciliated cells (Myerburg *et al*., 2007). Cilia are part of the innate immune defence of

airways *in vivo* and since ciliary activity is known to be compromised in CF, this is therefore one of the essential characteristics to be demonstrated in a valid model of CF airways.

This overall positive influence of fibroblasts on epithelial cell differentiation has been reported before. Myerburg *et al*. has shown for primary bronchial epithelial cells and lung fibroblasts, which were seeded on the undersurface of the TWs that this close proximity is necessary for promoting certain epithelial cell features. In this culture set up epithelial cells differentiated into a polarized ciliated cell layer, when intercellular contacts of epithelial cells and fibroblasts were possible but when direct cellular contact was prevented, by seeding fibroblasts at the bottom of the well that holds the TW, the epithelial cells differentiated into non-physiological, stratified squamous epithelium. This underlines that epithelial cell interactions with fibroblasts are important for normal cell differentiation (Myerburg *et al*., 2007). This study clearly shows that a close proximity of fibroblasts and epithelial cells is important for epithelial differentiation. Even though fibroblast conditioned media has been shown to enhance cell proliferation similarly to fibroblasts, proliferation and differentiation are two different things (Skibinski *et al*., 2007). Again this shows that fibroblast contact with epithelial cells needs to be further investigated and it cannot be assumed that based on the fact that proliferation is promoted by conditioned medium and fibroblasts to the same extent that this is the same for other cell function and developments as shown by Myerburg *et al*. (2007) for differentiation. The close proximity and direct cell contacts are definitely provided in the co-culture models presented in this work as fibroblasts were seeded directly into the TW as subepithelial cell layer, rather than on the undersurface of the membrane. This gives fibroblasts and epithelial cells chance to communicate closely, indeed, they are in direct contact. These co-culture models will be helpful for further elucidating these essential communications.

Some other co-culture models have been developed for airway research, which also apply fibroblasts into their models. One model for airway wall remodelling was developed by Choe *et al*. (2003). In this model fibroblasts are seeded and cultured in a collagen matrix and the epithelial cells are in a separate device that can be put on top of the fibroblasts, which creates a certain physical barrier between them but growth factors and other secreted macromolecules can diffuse through the model. This group has managed to develop a dynamic cell model for mechanical stress in the airways for research of remodelling in asthma (Choe *et al*., 2006), however as mentioned above direct cell contacts between epithelial cells and fibroblasts lead to different results in terms of epithelial cell differentiation suggesting that hepatocyte growth

factor and other fibroblast secretions are not the only influence on epithelial cell phenotypes (Myerburg *et al*., 2007).

Another model system that was established for CF airway research applied post-mitotic fibroblasts in the co-culture system with primary epithelial cells isolated from patients. The fibroblasts in these models were included with the main intention to keep primary cultures growing for longer based on the promoting effects of fibroblasts, and to establish more cultures out of one tissue sample of a patient, as these are limited and not always readily available. Post-mitotic fibroblasts were generated by mitomycin C treatment, these were then used as feeder layers seeded on the undersurface of TWs. Whilst this gives the cells chance to partially interact directly through the membrane there is still a physical barrier between them. Even though this group has shown that post-mitotic fibroblasts were more efficient in keeping the differentiated epithelium growing compared to conditioned medium only (Wiszniewski *et al*., 2006) there is room to criticise this approach at least the intention to use these models for CF research in terms of inflammatory responses because mitomycin C has been shown to increase IL-8 secretion in corneal fibroblasts for example (Chou *et al*., 2007). As IL-8 is one of the major chemokines found in CF airways, this attribute should not be manipulated if the models are to retain the most relevant features of the disease. Furthermore it was shown that mitomycin-treated fibroblasts are still able to express cytokine but whether this is true for ECM components for example is not known. These reasons underline the importance for the use of actively proliferating fibroblasts and enable to investigate the true pro-inflammatory responses by these cells.

The biggest concern when using co-culture models is that the fibroblasts will continue to proliferate and eventually overgrow the epithelial cells. However, it has been shown here (figure 4.12) that the fibroblasts grow subepithelial without overgrowing the epithelium and that co-cultures are established as two distinct populations of cells. Previous reports demonstrated that primary human bronchial fibroblasts expanded by threefold in 10-12 days and therefore had to be mitomycin C treated to prevent over-proliferation (Skibinski *et al*., 2007), here it has been shown not to happen with primary human pulmonary fibroblasts. Epithelial cell surface analysis using SEM confirms that there are two cell populations and that fibroblasts remain most proximal to the TW membrane, as there were no fibroblasts visible on the co-culture surface (figures 4.13, 4.16, 4.18, 4.22).

All together the epithelial cell features that were analysed in mono-cultures could all be identified in co-cultures as well but the addition of fibroblasts creates a model that is more relevant to *in vivo* physiology. As it has been shown before that fibroblasts stimulate epithelial cell growth (Skibinski *et al*., 2007) and epithelial cell differentiation (Myerburg *et al*., 2007)

The precise component(s) provided by actively proliferating fibroblasts that may support epithelial cell differentiation in co-culture models remain to be determined. However, one relevant growth factor that is usually secreted by fibroblasts is HGF and this growth factor has been shown to support epithelial cell proliferation (Skibinski *et al*., 2007) and differentiation (Myerburg *et al*., 2007). Furthermore for CF it has been suggested that the increased level of HGF found in BAL, is an indicator of actively ongoing repair mechanisms in CF and that this increased level of active HGF is responsible for the appearance of a relative normal epithelium with limited fibrotic changes (Shute *et al*., 2003). Fibrotic changes are not very apparent in CF but as suggested here, it is because of subepithelial fibroblasts that secrete HGF and therefore this fibroblastic support seems to be very important in the disease settings.

Whilst there is abundant evidence for the importance of fibroblasts in a model of the normal airways, this is not true for CF airways since almost no significant fibrotic changes are observed in CF airways *in vivo*. There are still other fibroblast functions, such as growth factor secretion, which should be taken into account when modelling the disease and it is most likely that these cells play a role in inflammatory responses, which will be discussed further on.

9.1.4 Inflammatory responses are different in mono-and co-cultures

Having established promising non-CF and CF mono and co-culture models these were challenged with LPS, live bacteria and HIA bacteria to observe their inflammatory responses and to validate how these results are in accordance with other existing models. Additionally these models will enable further investigation of the fibroblastic influence on inflammatory responses in CF lung disease and their impact on epithelial cells.

Submerged cell mono-cultures were analysed for cell viability and IL-8 induction after the three challenges with bacteria (LPS, HIA, live bacteria). The submerged cultures were the simplest cell culture models used in this project, and they were found to respond completely differently to these challenges compared to cell cultures at ALI. For example C38 responded to *B. cepacia* LPS under submerged conditions but not when cultured at ALI and neither did HPF-C38. As 3D cell culture mimics the *in vivo* cell morphology closely, such as the pseudostratified like appearance, it is suggested here that the inflammatory response is more similar in ALI mono- and co-cultures compared to *in vivo*. The fact that submerged cultures respond to bacterial challenges, whereas ALI cultures do not is at least partially down to the lack of polarity in these submerged cultures, which were undifferentiated mono-layers. These mono-layers do not form a tight epithelial cell layer, which has been shown by staining of the tight junction protein ZO-1. In undifferentiated submerged cultures ZO-1 is expressed but the typical belt-like staining pattern around the cells is lacking (figure 5.4). Therefore a distinct apical and basolateral membrane is not present and no apically directed secretions could be retained on the upper side of the cells.

Here it was shown that C38 and IB3-1's apical secretions only contained MUC5AC after ALI was established, suggesting that a protective mucus layer with possibly antibacterial activity is secreted and is suggested to prevent LPS from stimulating an inflammatory response in ALI cultures as it is likely that the LPS will get entrapped by the mucus layer. For Calu-3 it has been shown before that there is significant antibacterial activity in apical secretions of Calu-3 mono-cultures. The reported data show that *P. aeruginosa* and *E. coli* grown in apical secretions were killed to a certain extent, depending on the initial bacteria load added to the secretions (Zhang *et al*., 2001b).

In general it was observed that, following culture at ALI and therefore the formation of a polarised, differentiated epithelial cell layer, mono-cultures responded differently compared to co-cultures. This is not surprising considering that HPF add more complexity to the model and are likely to modulate cell behaviour and pro-inflammatory responses. Of course epithelial cells

are able to communicate with HPF and vice versa via signalling molecules such as growth factors, for example.

9.1.5 Fibroblasts respond to infectious stimuli

Direct cell–cell interactions, as discussed earlier are important in terms of fibroblast-epithelial cell communication and were shown to support epithelial cell proliferation (Skibinski *et al*., 2007) and differentiation (Myerburg *et al*., 2007) in different co-culture systems. Other important cell-cell communications between fibroblasts and other immune cells have also been reported. Alveolar macrophages, for example, secrete TNF-α in response to inflammation. This has been reported to induce IL-8 secretion in fibroblasts and therefore it was suggested that an initial host response is necessary to stimulate HPF (Rolfe *et al*., 1991). Data presented here show that fibroblasts can respond *directly* to inflammatory stimuli and secrete IL-8. In the airways, fibroblasts would not normally encounter bacteria and other pathogens, being located subepithelial, but after injury or bacterial invasion of the epithelium by intact bacteria this could be different and lead to direct contact between these pathogens and the fibroblasts. The three CF-relevant pathogens used in the current work have all been shown to be able to disrupt tight junctions and invade the epithelium (da Silva *et al*., 2004, Kim *et al*., 2005b, Azghani, 1996). In TW mono-cultures, HPF respond to all live bacteria used in these experiments with IL-8 secretion to the apical and basolateral side, suggesting that they will have an influence on the overall inflammation found *in vivo*. Here IL-8 was found in the apical compartment, which is suggested to maintain the luminal chemokine concentration that would keep neutrophils at the side of infection plus IL-8 was found in the basolateral compartment, where it has been shown before that signalling to the underlying tissue cells occurs to recruit neutrophils to the site of infection (Conese *et al*., 2003).

Another interesting point to note is that live *S. aureus* decreased cell viability in C38 and IB3-1 mono-cultures and also in co-cultures but with the difference that IL-8 is only induced in co-cultures. This underlines that there is a different inflammatory response in mono- and co-cultures. As already discussed in chapter 7, *S. aureus* is able to invade epithelial cells and induce apoptosis or cause necrosis (Essmann *et al*., 2003), suggesting that in mono-cultures predominantly apoptosis occurs, which does not cause a pro-inflammatory response and that this causes the lack of inflammatory response. However, HPF mono-cultures have been shown to respond to live *S. aureus* following the apical and the apical/basolateral challenge and similar responses were seen for HPF-C38 (following apical/basolateral) and HPF-IB3-1 (apical, basal), which shows that either the fibroblasts respond directly by secreting IL-8,when stimulated from

the basolateral side or in case of apical challenge that *S. aureus* has possibly invaded the epithelial cell layer and traverse to the subepithelial cell layer to induce IL-8 secretion.

Results for live *B. cepacia* showed that the cell viability was only affected in co-cultures but not in mono-cultures of C38 , IB3-1 or HPF. This indicates that co-cultures are more susceptible to this bacterium. Whether the decrease in cell viability is down to dying fibroblasts, to dying epithelial cells or to both cell types cannot be indentified at this point. HIA *B. cepacia* is not able to decrease viability at all, which indicates that only live *B. cepacia* causes this damage, perhaps via invasion of the epithelium. The invasion of lung tissue with *B. cepacia* has been shown before in tissue samples of CF patients (Sajjan *et al*., 2001) as well as for epithelial cells (Martin and Mohr, 2000). Interestingly it was observed here that HPF-IB3-1 were affected to a higher extent compared to HPF-C38 and cell viability was decreased following all three challenges in HPF-IB3-1 compared to only following the simultaneous challenge from both compartments in HPF-C38. This suggests that *B. cepacia* was able to invade IB3-1 cells in the co-culture model from the apical side but was not able to do so in C38 following the apical challenge. It has been shown before that several strains of *B. cepacia*, which express cable-pilus, were able to bind to CF cells from human lung sections but not to non-CF ones. The same was observed for mice nasal tissue sections (Sajjan *et al*., 2000a).These findings support what was observed here that HPF-IB3-1 are more susceptible to this challenge than HPF-C38. Even though in Sajjan *et al*. (2000) no binding of *B. cepacia* was seen to non-CF cells at all this is not a direct contrast to the decreased viability seen in HPF-C38 following the simultaneous challenge, as they only analysed epithelial cells, whereas the cell viability decrease of HPF-C38 could be explained as a result of bacterial binding to and/or invading the fibroblasts. A possible receptor that *B. cepacia* are known to bind to is TNFR1 (Sajjan *et al*., 2008), which is expressed in fibroblasts an could therefore lead to the cytotoxicity observed in the co-cultures.

Comparing pro-inflammatory responses, in terms of IL-8 release, following the challenge with HIA *B. cepacia* and intact *B. cepacia* it becomes clear that only the viable form of *B. cepacia* is able to induce a pro-inflammatory response in these mono- and co-cultures of HPF, C38 and IB3-1. This is not surprising considering that HIA bacteria are targeted to the lysosome usually within 4 h post infection, whereas live *B. cepacia* have been shown to escape late endosomes and lysosomes to enter autophagosomes and replicate afterwards within the endoplasmatic reticulum (Sajjan *et al*., 2006).

In case of cultures challenged with either live *P. aeruginosa* or live *S. aureus* cell viability is affected in mono-and co-cultures but to a greater extent in mono-cultures, which is in contrast to what was observed following challenges with *B. cepacia*, after which only the co-cultures

showed significant decrease in cell viability. Following *P. aeruginosa* challenges, the cell viability of IB3-1 mono-cultures was reduced by 27 % but was not affected in the co-culture system after the basal challenge, suggesting some kind of protection given by the HPF cell layer. This result was also observed following the challenge with live *S. aureus*. Interestingly in a different setting it has been shown that fibroblasts can actively support cell survival and it was shown that fibroblasts and their secreted ECM components plus growth factors supported cell viability of pancreatic islets (Jalili *et al*., 2010). Further investigation is needed here to get an insight into this possible protection by fibroblasts. That this possibly protective layer is not able to defend the co-cultures against live *B. cepacia* on the basolateral side indicates the strong virulence of this bacterium, especially once it has invaded the cells. *B. cepacia* are known to be the most invasive infection found in CF patients and usually leads to a rapid decline in lung function and often to septicaemia (Mahenthiralingam *et al*., 2005).

In the case of challenges with live *P. aeruginosa*, basal challenge induced IL-8 secretion by the HPF-C38 and HPF-IB3-1 co-cultures but surprisingly none of the mono-cultures of HPF, C38 or IB3-1 responded to this challenge by secreting IL-8. Again there were completely different responses seen in mono- and co-cultures, which is most likely due to cell-cell communications between the epithelial cells and fibroblasts. As the stimulus was given basolaterally it is possible a signalling cascade starts with pathogen binding to HPF, which then respond by release of a mediator other than IL-8, which is able to stimulate the overlying epithelial cells to apically secrete IL-8. It is not clear if HPF respond only to certain bacterial stimuli, for example different virulence factors, by secreting IL-8 and whether they respond differently to other virulence factors, which might induce different signalling pathways leading to different mediators.
Even though no underlying mechanism for these differences in IL-8 responses of mono- and co-cultures can currently be defined, these differences highlight again that fibroblasts and cell-cell interactions are important in bacterial induced inflammatory responses. Again this underlines the importance of taking fibroblasts into account to further study CF lung pathogenesis and that mono-cultures studied only show small part of their contribution to the overall IL-8 driven neutrophilic inflammation seen in CF.

Different pro-inflammatory responses were observed for mono- and co-cultures following infection with three different CF-relevant organisms. For the challenges with live bacteria it needs to be kept in mind that all these bacteria work to a great extent by the regulation of their quorum sensing signalling systems and they all secrete different virulence factors (Yarwood and Schlievert, 2003, McKeon *et al*., 2011, Erickson *et al*., 2002). Furthermore *S. aureus* is a gram

positive bacterium and the other two bacteria are gram negative, which will have an influence of the cellular response as different signalling pathways (via, for example, distinct TLRs) will be activated by different stimuli to induce IL-8 secretion from epithelial cells (Greene *et al*., 2005a, John *et al*., 2010). As earlier discussed in this work (chapter six) it was shown that none of these cells express TLR4 on their membrane surface but are able to respond to LPS. This mechanism has not been fully elucidated yet but it has been reported before that intracellular TLR-4 can be located in the Golgi apparatus and there it co-localizes with internalized LPS in intestinal epithelial cells for example (Hornef *et al*., 2002), which could also be a possible control mechanism, so that airway epithelial cells do not respond to low levels of LPS or even of the bacterial products as these are inhaled constantly but they must be able to respond to a certain load of LPS or live bacteria to be able to respond quickly and eliminate the infection. As this is a more complex cell-culture model and at least in the case of using live bacteria there are lots of pathways involved, such as TLRs (Muir *et al*., 2004, Greene *et al*., 2005b) and asialoGM1 (DiMango *et al*., 1995, Kube *et al*., 2001), which can both modulate the interaction with bacteria these experiments need future investigation to elucidate the dynamic interactions of epithelial cells with fibroblasts and vice versa but also the dynamic interactions of fibroblasts or epithelial cells with bacteria. Fibroblasts have not been extensively studied in terms of inflammation in CF but as shown here are able to respond to bacterial infections and therefore must have receptors, such as TLRs and TNFR1, that initiate a signalling pathway to induce IL-8 expression.

9.1.6 A novel co-culture model mimicking CF

The overall aim was to establish co-culture systems for non-CF and CF human airways *in vitro* using HPF and one of the epithelial cell lines C38, IB3-1 or Calu-3. As earlier discussed Calu-3 are not useful as a control for IB3-1 and will therefore not be taken forward as a suitable non-CF model of human airways. In mono-cultures of C38 and IB3-1 it was observed that the TER of IB3-1 was in general higher compared to C38. However the difference was not significant. When these epithelial cells were co-cultured with HPF this changed and the TER was significantly higher in the CF model. This is in accordance with other reports that have shown that epithelial TER in CF cell is higher based on the dysfunction of CFTR. It was also shown by the same group that using the CFTR-inhibitor $CFTR_{inh}$-172 on normal cells (16HBE) had the same affect in terms of increasing TER (Nilsson *et al*., 2010, Perez *et al*., 2007).

Furthermore it has been observed here that IB3-1 did not constantly secrete higher IL-8 levels at baseline, which is in contrast to what has been shown before (DiMango *et al*., 1998) , however,

the magnitude of IL-8 response is always greater in the CF models. There is an existing area of controversy about constitutively increased IL-8 in CF and which leads to one of the outstanding questions in CF: What comes first inflammation or infection?

It has been reported that even without infection in CF infants there was an increased IL-8 concentration in the BAL fluid in comparison with control subjects. This was also shown for neutrophil counts and neutrophil elastase (Khan *et al*., 1995), which indicates that inflammation is present before infection. However there are other reports that show that CF and non-CF cells secrete similar amounts of IL-8 (Kube *et al*., 2001, Scheid *et al*., 2001) but that CF cells show a hyperinflammatory response after exposure to *P. aeruginosa*, which was appointed to the fact that there is increased bacterial adherence to CF cells. These reports are in accordance with the hyperinflammatory state found here for challenged IB3-1 cells in mono- and co-cultures.

In this study here it was observed that IB3-1 cells respond with much higher secretion of IL-8 to the live bacteria used. After the challenge with live *P. aeruginosa* the IL-8 concentration in apical supernatants (following apical or apical/basolateral challenges) was about 2.5-fold higher in IB3-1 samples compared to C38. This difference was even higher after *B. cepacia* challenges, where IL-8 was found to be 3-fold higher for IB3-1 samples. Importantly the typical hyperinflammatory state which is reported for CF, is still present in co-cultures. After exposure to live *P. aeruginosa*, the IL-8 concentration in apical supernatants of IB3-1 was still 1.5-fold higher compared to HPF-C38. For the apical/basolateral challenge it is not possible to make this statement as HPF-IB3-1 cell viability was reduced by 67 % and this does not allow an accurate comparison to HPF-C38, whose cell viability was only decreased by 33 %.

These decreases in cell viabilities, directs to the next CF phenotypic feature presented in this model, hypersusceptibility to infection. There were several different approaches that tried to find the direct link to why CFTR loss or mutation causes such severe lung disease in most patients (depending on the CFTR mutation). CF cells have been closely studied and one receptor that has been found to be more numerous on CF cells compared to non-CF cells is asialoGM1 (DiMango *et al*., 1995). This receptor is a binding site for *P. aeruginosa* pili and flagellin, for example (Schroeder *et al*., 2001b) and therefore it was suggested to be involved in the hypersusceptibility of CF patients to *P. aeruginosa* infections by higher adherence of the bacteria to CF cells. Although this receptor is more prominent on IB3-1 cells (DiMango *et al*., 1995), the *P. aeruginosa* strain used here, was isolated from a patient and therefore most likely turned into a non-motile bacterium and does not express flagella anymore (de Bentzmann *et al*., 1996b, de Bentzmann *et al*., 1996a, Mahenthiralingam *et al*., 1994). However the susceptibility seemed to be higher in IB3-1 models compared to C38 models but the precise mechanism remains to be

determined as there are a variability of other factors that need to be taken into account, such as the difference of mucus composition in CF and non-CF (Roussel *et al*., 1975, Thornton and Sheehan, 2004).

These co-culture models display CF phenotypic features and are promising co-culture models for further investigations, especially with the integration of actively proliferating fibroblasts, which will help to shed more light on the role these cells might play in CF lung disease and the overall pro-inflammatory state found in CF. These models are still in their infancies but they show that fibroblasts are more than just feeder layers and they will be a useful addition to existing models, especially a good addition to the few co-culture models that have been established for CF research.

9.1.7 CF models – aren't there enough???

The answer simply is NO. It is important that new and improved models are developed because the investigation of lung pathogenesis has been partially hampered by the necessary but not existing models. Novel models will help to further understand CF pathogenesis and in the long term help to increase life expectancies and to improve the quality of life for CF patients by developing new therapies based on research findings.

In this project novel co-culture models were established for mimicking CF and non-CF human bronchial airways *in vitro* and they have been shown throughout this work to fulfil essential characteristic expectations and they have been shown to show CF related differences between them. These are promising novel models and they not only look at epithelial cells and how they behave but also at subepithelial fibroblasts. These models are more complex compared to the standard used mono-cultures systems but they are much simpler compared to whole animal models, which gives them the advantage to be easier to control. As one of the future aims is to further investigate intercellular communication and investigate the underlying mechanisms, these models have the advantage compared to animal models that less variables are present and it should be easier to control experimental conditions and that is one reason for the need of additional *in vitro models*.

Even though, many mouse models have been developed for CF research and these show dramatic differences to the disease phenotypes compared to what the hallmarks are in human CF. As already pointed out in chapter 1 mice show a complete different lung anatomy compared to humans (Zollner *et al*., 2004) and most importantly mice do not express IL-8 and no clear cut

IL-8 homolog has been identified despite intensive research (Rovai *et al*., 1998). Two chemokines that are expressed by mice have been referred to as homologous to the human IL-8. One is the macrophage inflammatory protein 2-α (MIP-2) and the other one is keratinocyte chemoattractant (KC), which have both been shown to recruit neutrophils. These differences make it almost impossible to directly and accurately compare results found in human *in vitro* models and *in vivo* mouse models. The difference of chemokine expression and also the fact that humans express two IL-8 receptors and mice only express one KC/MIP receptor, show that experimental outcomes have to be taken with care, when these are tried to be related to the human disease (Zhang *et al*., 2001a). In addition it has been shown that the pathophysiology of the airway epithelium, which is one of the most affected areas in human lungs with CF, is not present in the animal models. Instead the animal models are dominated by the intestinal pathology, indeed, this is the main cause of morbidity in CF mice (Davidson and Dorin, 2001). In these CF mice no gross pulmonary differences were observed compared to their non-CF littermates. There is long list of differences, which have been reviewed extensively but here it was important to point out that a direct comparison of results found here for inflammation would not be very relevant and would not help in further investigating the inflammatory responses, for example, observed here.

There are some studies, which employ chronic infection of mouse models with *P. aeruginosa,* for example and they have shown that after a three day infection with *P. aeruginosa,* concentrations of the IL-8 homologues MIP-2 and KC in the BAL fluid were significantly increased compared to normal littermates (Heeckeren *et al*., 1997) but as bacteria have been shown to not be retained in mouse lungs these have to be embedded in agar beads, for example and therefore represent an artifical, exaggerated mode of infection. Hypersecretion of IL-8 in response to infection was observed in the mono- and co-culture models presented in this work and the IL-8 secretion of IB3-1 in mono-culture is about 3 times as high compared to C38. However, these comparisons are better to be made comparing human systems to each other as *in vitro* experimental settings are completely different and there are less variables presented compared to *in vivo*. As discussed earlier on, there is even criticism towards comparing different epithelial cell lines all having human origin and maybe originate from different parts of the respiratory system but the question arising then is: If human cell systems cannot be compared to each other, how can it be valuable to compare results to mouse models that do not even show the same pathophysiology? Therefore it is not the intention here to discuss outcomes from other mouse models.

9.1.8 Future work

Future work will include additional investigation of the presented model systems in terms of paraffin embedded cross-sections. As there is no histology department at Aston University the intention is to integrate these techniques into the laboratory. As it has been addressed earlier on, immunofluorescence staining on excised TWs, which were then analysed using fluorescent microscopy by viewing them from the apical side, is a good method for mono-cultures and antibody titrations, for example, but paraffin sections will be more useful in terms of co-culture characterisation. These paraffin cross-sections will enable further comparisons of the CF and the non-CF model in mono- and co-cultures following staining for different receptors, for example TLR expression. Unstimulated submerged cells have been analysed quite well but it is of great interest to further investigate the surface expression of different TLRs in response to different stimuli and when grown at ALI. This is also very important to further characterise the human pulmonary fibroblasts used here as this type of cell has not been extensively studied in the setting of CF lung disease. Furthermore goblet cells and mucin expression could be identified in this way and most importantly investigate the cultures before and after bacterial infections.

Another future aim is to further investigate mucin expression of these epithelial cell lines. It would be interesting to analyse, whether MUC5B is expressed and secreted by these epithelial cells. Quantitative analysis of mucus secretion has never been carried out in this laboratory but is another future aim to be addressed. This would be very useful as obviously it would be interesting to observe if IB3-1 hypersecrete mucus at baseline levels and what direct influence bacterial infections have on these cultures. Furthermore the analysis of mucus secretion in co-cultures of HPF and these epithelial cell lines would be interesting to investigate the subepithelial HPF affect on mucus secretion.

Additionally it is important to further investigate the role of HPF in these co-culture models in terms of ECM deposition, cytokine (other than IL-8) and growth factor secretion for example. ECM deposition by fibroblasts has been shown before (Skibinski *et al.*, 2007) and it would be interesting to see if ECM components are accumulated over time in these long-term cultures or whether some kind of equilibrium can be established over time between degradation and de novo synthesis.

In terms of epithelial cell characterisation and functionality there are further possibilities as well especially observing differences of mono-and co-cultures as well as observing possible differences based on being CF and non-CF. Another close future objective would be to further analyse MMPs in these culture models as some MMPs, such as MMP-2 have been shown to be increased in CF but in not much research has been carried out to investigate, which role this proteolytic enzyme might play in CF. This would include the analysis of MMPs and also the analysis of the tissue inhibitors of metalloproteinases (TIMPs). Again this is also an experiment to be analysed before and after infection with several CF relevant pathogens.

Another aspect is the investigation of antimicrobial properties presented in the ASL secretions of mono-and co-cultures and further analyse single components, which might be altered down to the epithelial difference being CF and non-CF and further what influence HPF have on these secretion, for example volume and antibacterial property changes, in the co-culture systems.

Chemotaxis assays will be carried out to detect if the IL-8 analysed at baseline and following infections is biologically active and able to attract neutrophils using a chemotaxis chamber.

An exposure time of 24 h was used here for the bacterial infections and this only gives a snapshot of one time point but it would be nice in terms of TER measurements, for example to carry out a time course throughout the infection to see whether there are changes to be noticed in between and when changes start to happen in response to certain bacteria. It could also be helpful to expose mono- and co-cultures cells to bacteria for different amount of time and then follow these developments. These time courses would also be helpful to investigate the cell viability changes found here in response to certain bacteria and observe when cells start dying (caspase activity) and if the culture can recover after gentamicin treatment. How long would these cultures survive in presence of infection?

Here three CF relevant bacteria were used separately to stimulate the mono- and co-cultures but in CF patients, even though *P. aeruginosa* is the most prominent bacterium, usually polymicrobial infections were observed, with new strains often being isolated and bacteria that have never been seen before in CF (CF trust). This is an interesting research field and if through possible collaborations (Heart of England, for example) new relevant pathogens could be received, these could be investigated using these mono- and co-culture models. There is different ways of approaching these kinds of experiments depending on what the main interest is. Cultures could be exposed to one bacterium first (*S. aureus*) and then after elimination another infection with *P. aeruginosa* could follow and see whether in these settings *S. aureus* has a promoting affect on *P. aeruginosa* infection. Furthermore polymicrobial infections of these

models could be carried out and investigate whether *P. aeruginosa* is the leading pathogen in these settings and further investigate the establishment of these bacterial communities in CF compared to non-CF.

Another long term aim will be the attempt to expand the established co-culture models by adding another dimension to it in terms of an additional cell type, such as endothelial cell or macrophages, for example to get another step closer to mimic normal *in vivo* physiology.

10 References

ABDULLAH, L. H. & DAVIS, C. W. 2007. Regulation of airway goblet cell mucin secretion by tyrosine phosphorylation signaling pathways. *Am J Physiol Lung Cell Mol Physiol,* 293**,** L591-9.

AKIRA, S. & TAKEDA, K. 2004. Toll-like receptor signalling. *Nat Rev Immunol,* 4**,** 499-511.

ALTON, E. W., MIDDLETON, P. G., CAPLEN, N. J., SMITH, S. N., STEEL, D. M., MUNKONGE, F. M., JEFFERY, P. K., GEDDES, D. M., HART, S. L., WILLIAMSON, R. & *ET AL*. 1993. Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice. *Nature genetics,* 5**,** 135-42.

ALTON, E. W., STERN, M., FARLEY, R., JAFFE, A., CHADWICK, S. L., PHILLIPS, J., DAVIES, J., SMITH, S. N., BROWNING, J., DAVIES, M. G., HODSON, M. E., DURHAM, S. R., LI, D., JEFFERY, P. K., SCALLAN, M., BALFOUR, R., EASTMAN, S. J., CHENG, S. H., SMITH, A. E., MEEKER, D. & GEDDES, D. M. 1999. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet,* 353**,** 947-54.

ARMSTRONG, D. S., GRIMWOOD, K., CARZINO, R., CARLIN, J. B., OLINSKY, A. & PHELAN, P. D. 1995. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. *BMJ,* 310**,** 1571-2.

ATSUTA, J., STERBINSKY, S. A., PLITT, J., SCHWIEBERT, L. M., BOCHNER, B. S. & SCHLEIMER, R. P. 1997. Phenotyping and cytokine regulation of the BEAS-2B human bronchial epithelial cell: demonstration of inducible expression of the adhesion molecules VCAM-1 and ICAM-1. *Am J Respir Cell Mol Biol,* 17**,** 571-82.

AUMAILLEY, M. & TIMPL, R. 1986. Attachment of cells to basement membrane collagen type IV. *J Cell Biol,* 103**,** 1569-75.

AZGHANI, A. O. 1996. Pseudomonas aeruginosa and epithelial permeability: role of virulence factors elastase and exotoxin A. *American journal of respiratory cell and molecular biology,* 15**,** 132-40.

AZGHANI, A. O., KONDEPUDI, A. Y. & JOHNSON, A. R. 1992. Interaction of Pseudomonas aeruginosa with human lung fibroblasts: role of bacterial elastase. *American journal of respiratory cell and molecular biology,* 6**,** 652-7.

BAMFORD, S., RYLEY, H. & JACKSON, S. K. 2007. Highly purified lipopolysaccharides from Burkholderia cepacia complex clinical isolates induce inflammatory cytokine responses via TLR4-mediated MAPK signalling pathways and activation of NFkappaB. *Cellular microbiology,* 9**,** 532-43.

BAUMANN, U., KING, M., APP, E. M., TAI, S., KONIG, A., FISCHER, J. J., ZIMMERMANN, T., SEXTRO, W. & VON DER HARDT, H. 2004. Long term azithromycin therapy in cystic fibrosis patients: a study on drug levels and sputum properties. *Canadian respiratory journal : journal of the Canadian Thoracic Society,* 11**,** 151-5.

BAUTISTA, M. V., CHEN, Y., IVANOVA, V. S., RAHIMI, M. K., WATSON, A. M. & ROSE, M. C. 2009. IL-8 regulates mucin gene expression at the posttranscriptional level in lung epithelial cells. *J Immunol,* 183**,** 2159-66.

BECKER, M. N., SAUER, M. S., MUHLEBACH, M. S., HIRSH, A. J., WU, Q., VERGHESE, M. W. & RANDELL, S. H. 2004. Cytokine secretion by cystic fibrosis airway epithelial cells. *Am J Respir Crit Care Med,* 169**,** 645-53.

BERNACKI, S. H., NELSON, A. L., ABDULLAH, L., SHEEHAN, J. K., HARRIS, A., DAVIS, C. W. & RANDELL, S. H. 1999. Mucin gene expression during differentiation of human airway epithelia *in vitro*. Muc4 and muc5b are strongly induced. *American journal of respiratory cell and molecular biology,* 20**,** 595-604.

BONFIELD, T. L., PANUSKA, J. R., KONSTAN, M. W., HILLIARD, K. A., HILLIARD, J. B., GHNAIM, H. & BERGER, M. 1995. Inflammatory cytokines in cystic fibrosis lungs. *American journal of respiratory and critical care medicine,* 152**,** 2111-8.

BOSCH, F. X., LEUBE, R. E., ACHTSTATTER, T., MOLL, R. & FRANKE, W. W. 1988. Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. *The Journal of cell biology,* 106**,** 1635-48.

BOUCHER, R. C. 2007. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. *Trends Mol Med,* 13**,** 231-40.

BREEZE, R. G. & WHEELDON, E. B. 1977. The cells of the pulmonary airways. *The American review of respiratory disease,* 116**,** 705-77.

BREWSTER, C. E., HOWARTH, P. H., DJUKANOVIC, R., WILSON, J., HOLGATE, S. T. & ROCHE, W. R. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *American journal of respiratory cell and molecular biology,* 3**,** 507-11.

BUCHANAN, J. A., YEGER, H., TABCHARANI, J. A., JENSEN, T. J., AUERBACH, W., HANRAHAN, J. W., RIODAN, J. R. & BUCHWALD, M. 1990. Transformed sweat gland and nasal epithelial cell lines from control and cystic fibrosis individuals. *Journal of cell science,* 95 (Pt 1)**,** 109-23.

BURGEL, P. R. & NADEL, J. A. 2004. Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. *Thorax,* 59**,** 992-6.

BURNS, J. L., EMERSON, J., STAPP, J. R., YIM, D. L., KRZEWINSKI, J., LOUDEN, L., RAMSEY, B. W. & CLAUSEN, C. R. 1998. Microbiology of sputum from patients at cystic fibrosis centers in the United States. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 27**,** 158-63.

CAPLEN, N. J., ALTON, E. W., MIDDLETON, P. G., DORIN, J. R., STEVENSON, B. J., GAO, X., DURHAM, S. R., JEFFERY, P. K., HODSON, M. E., COUTELLE, C. & *ET AL*. 1995. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature medicine,* 1**,** 39-46.

CASH, H. A., WOODS, D. E., MCCULLOUGH, B., JOHANSON, W. G., JR. & BASS, J. A. 1979. A rat model of chronic respiratory infection with Pseudomonas aeruginosa. *The American review of respiratory disease,* 119**,** 453-9.

CHOE, M. M., SPORN, P. H. & SWARTZ, M. A. 2006. Extracellular matrix remodeling by dynamic strain in a three-dimensional tissue-engineered human airway wall model. *Am J Respir Cell Mol Biol,* 35**,** 306-13.

CHOE, M. M., SPORN, P. H. S. & SWARTZ, M. A. 2003. An *in vitro* airway wall model of remodeling. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 285**,** L427-L433.

CHOU, S. F., CHANG, S. W. & CHUANG, J. L. 2007. Mitomycin C upregulates IL-8 and MCP-1 chemokine expression via mitogen-activated protein kinases in corneal fibroblasts. *Investigative ophthalmology & visual science,* 48**,** 2009-16.

CHOW, A. W., LIANG, J. F., WONG, J. S., FU, Y., TANG, N. L. & KO, W. H. 2010. Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o- cells in response to cationic polypeptide challenge. *PLoS One,* 5**,** e12091.

CIGANA, C., CURCURU, L., LEONE, M. R., IERANO, T., LORE, N. I., BIANCONI, I., SILIPO, A., COZZOLINO, F., LANZETTA, R., MOLINARO, A., BERNARDINI, M. L. & BRAGONZI, A. 2009. Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PLoS One,* 4**,** e8439.

CLAUDE, P. & GOODENOUGH, D. A. 1973. Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *The Journal of cell biology,* 58**,** 390-400.

CLUNES, M. T. & BOUCHER, R. C. 2007. Cystic Fibrosis: The Mechanisms of Pathogenesis of an Inherited Lung Disorder. *Drug Discov Today Dis Mech,* 4**,** 63-72.

CONESE, M., COPRENI, E., DI GIOIA, S., DE RINALDIS, P. & FUMARULO, R. 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *J Cyst Fibros,* 2**,** 129-35.

CORAUX, C., ROUX, J., JOLLY, T. & BIREMBAUT, P. 2008. Epithelial cell-extracellular matrix interactions and stem cells in airway epithelial regeneration. *Proc Am Thorac Soc,* 5**,** 689-94.

COULOMBE, P. A., KOPAN, R. & FUCHS, E. 1989. Expression of keratin K14 in the epidermis and hair follicle: insights into complex programs of differentiation. *The Journal of cell biology,* 109**,** 2295-312.

COURTNEY, J. M., DUNBAR, K. E., MCDOWELL, A., MOORE, J. E., WARKE, T. J., STEVENSON, M. & ELBORN, J. S. 2004. Clinical outcome of Burkholderia cepacia complex infection in cystic fibrosis adults. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society,* 3**,** 93-8.

COZENS, A. L., YEZZI, M. J., KUNZELMANN, K., OHRUI, T., CHIN, L., ENG, K., FINKBEINER, W. E., WIDDICOMBE, J. H. & GRUENERT, D. C. 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *American journal of respiratory cell and molecular biology,* 10**,** 38-47.

CRYSTAL, R. G., MCELVANEY, N. G., ROSENFELD, M. A., CHU, C. S., MASTRANGELI, A., HAY, J. G., BRODY, S. L., JAFFE, H. A., EISSA, N. T. & DANEL, C. 1994. Administration of an adenovirus

containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nature genetics,* 8**,** 42-51.

CRYSTAL, R. G., RANDELL, S. H., ENGELHARDT, J. F., VOYNOW, J. & SUNDAY, M. E. 2008. Airway epithelial cells: current concepts and challenges. *Proceedings of the American Thoracic Society,* 5**,** 772-7.

DA SILVA, M. C., ZAHM, J. M., GRAS, D., BAJOLET, O., ABELY, M., HINNRASKY, J., MILLIOT, M., DE ASSIS, M. C., HOLOGNE, C., BONNET, N., MERTEN, M., PLOTKOWSKI, M. C. & PUCHELLE, E. 2004. Dynamic interaction between airway epithelial cells and Staphylococcus aureus. *American journal of physiology. Lung cellular and molecular physiology,* 287**,** L543-51.

DAKO 2006. *Education Guide, Immunohistochemical Staining Methods*.

DALEY, W. P., PETERS, S. B. & LARSEN, M. 2008. Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci,* 121**,** 255-64.

DANIEL, M. R. & BURNETT, H. E. 1991. Immunocytochemical investigation of the tissue of origin of two lung cancer cell lines. *International journal of experimental pathology,* 72**,** 397-405.

DAVIDSON, D. J. & DORIN, J. R. 2001. The CF mouse: an important tool for studying cystic fibrosis. *Expert reviews in molecular medicine,* 2001**,** 1-27.

DAVIDSON, D. J., DORIN, J. R., MCLACHLAN, G., RANALDI, V., LAMB, D., DOHERTY, C., GOVAN, J. & PORTEOUS, D. J. 1995. Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. *Nature genetics,* 9**,** 351-7.

DAVIES, J. C. & ALTON, E. W. 2010. Gene therapy for cystic fibrosis. *Proceedings of the American Thoracic Society,* 7**,** 408-14.

DE BENTZMANN, S., PLOTKOWSKI, C. & PUCHELLE, E. 1996a. Receptors in the Pseudomonas aeruginosa adherence to injured and repairing airway epithelium. *American journal of respiratory and critical care medicine,* 154**,** S155-62.

DE BENTZMANN, S., ROGER, P., DUPUIT, F., BAJOLET-LAUDINAT, O., FUCHEY, C., PLOTKOWSKI, M. C. & PUCHELLE, E. 1996b. Asialo GM1 is a receptor for Pseudomonas aeruginosa adherence to regenerating respiratory epithelial cells. *Infection and immunity,* 64**,** 1582-8.

DE BENTZMANN, S., ROGER, P. & PUCHELLE, E. 1996c. Pseudomonas aeruginosa adherence to remodelling respiratory epithelium. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology,* 9**,** 2145-50.

DELACOURT, C., LE BOURGEOIS, M., D'ORTHO, M. P., DOIT, C., SCHEINMANN, P., NAVARRO, J., HARF, A., HARTMANN, D. J. & LAFUMA, C. 1995. Imbalance between 95 kDa type IV collagenase and tissue inhibitor of metalloproteinases in sputum of patients with cystic fibrosis. *American journal of respiratory and critical care medicine,* 152**,** 765-74.

DELANEY, S. J., ALTON, E. W., SMITH, S. N., LUNN, D. P., FARLEY, R., LOVELOCK, P. K., THOMSON, S. A., HUME, D. A., LAMB, D., PORTEOUS, D. J., DORIN, J. R. & WAINWRIGHT, B. J. 1996.

Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *The EMBO journal,* 15**,** 955-63.

DELGADO, M. A., POSCHET, J. F. & DERETIC, V. 2006. Nonclassical pathway of Pseudomonas aeruginosa DNA-induced interleukin-8 secretion in cystic fibrosis airway epithelial cells. *Infect Immun,* 74**,** 2975-84.

DENNING, G. M., WOLLENWEBER, L. A., RAILSBACK, M. A., COX, C. D., STOLL, L. L. & BRITIGAN, B. E. 1998. Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infection and immunity,* 66**,** 5777-84.

DI SANT'AGNESE, P. A., DARLING, R. C., PERERA, G. A. & SHEA, E. 1953. Sweat electrolyte disturbances associated with childhood pancreatic disease. *The American journal of medicine,* 15**,** 777-84.

DIAZ, A., VARGA, J. & JIMENEZ, S. A. 1989. Transforming growth factor-beta stimulation of lung fibroblast prostaglandin E2 production. *J Biol Chem,* 264**,** 11554-7.

DIMANGO, E., RATNER, A. J., BRYAN, R., TABIBI, S. & PRINCE, A. 1998. Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *The Journal of clinical investigation,* 101**,** 2598-605.

DIMANGO, E., ZAR, H. J., BRYAN, R. & PRINCE, A. 1995. Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. *The Journal of clinical investigation,* 96**,** 2204-10.

DONALDSON, S. H., BENNETT, W. D., ZEMAN, K. L., KNOWLES, M. R., TARRAN, R. & BOUCHER, R. C. 2006. Mucus clearance and lung function in cystic fibrosis with hypertonic saline. *N Engl J Med,* 354**,** 241-50.

DORFMAN, R., LI, W., SUN, L., LIN, F., WANG, Y., SANDFORD, A., PARE, P. D., MCKAY, K., KAYSEROVA, H., PISKACKOVA, T., MACEK, M., CZERSKA, K., SANDS, D., TIDDENS, H., MARGARIT, S., REPETTO, G., SONTAG, M. K., ACCURSO, F. J., BLACKMAN, S., CUTTING, G. R., TSUI, L. C., COREY, M., DURIE, P., ZIELENSKI, J. & STRUG, L. J. 2009. Modifier gene study of meconium ileus in cystic fibrosis: statistical considerations and gene mapping results. *Human genetics,* 126**,** 763-78.

DORIN, J. R., DICKINSON, P., ALTON, E. W., SMITH, S. N., GEDDES, D. M., STEVENSON, B. J., KIMBER, W. L., FLEMING, S., CLARKE, A. R., HOOPER, M. L. & *ET AL*. 1992. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature,* 359**,** 211-5.

DREVINEK, P., HOLDEN, M. T., GE, Z., JONES, A. M., KETCHELL, I., GILL, R. T. & MAHENTHIRALINGAM, E. 2008. Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum. *BMC infectious diseases,* 8**,** 121.

DREVINEK, P. & MAHENTHIRALINGAM, E. 2010. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases,* 16**,** 821-30.

DRUMM, M. L., POPE, H. A., CLIFF, W. H., ROMMENS, J. M., MARVIN, S. A., TSUI, L. C., COLLINS, F. S., FRIZZELL, R. A. & WILSON, J. M. 1990. Correction of the cystic fibrosis defect *in vitro* by retrovirus-mediated gene transfer. *Cell,* 62**,** 1227-33.

DU, M., JONES, J. R., LANIER, J., KEELING, K. M., LINDSEY, J. R., TOUSSON, A., BEBOK, Z., WHITSETT, J. A., DEY, C. R., COLLEDGE, W. H., EVANS, M. J., SORSCHER, E. J. & BEDWELL, D. M. 2002. Aminoglycoside suppression of a premature stop mutation in a Cftr-/- mouse carrying a human CFTR-G542X transgene. *Journal of molecular medicine,* 80**,** 595-604.

DUNSMORE, S. E. & RANNELS, D. E. 1996. Extracellular matrix biology in the lung. *Am J Physiol,* 270**,** L3-27.

DUSZYK, M., SHU, Y., SAWICKI, G., RADOMSKI, A., MAN, S. F. & RADOMSKI, M. W. 1999. Inhibition of matrix metalloproteinase MMP-2 activates chloride current in human airway epithelial cells. *Canadian journal of physiology and pharmacology,* 77**,** 529-35.

ECKES, B., DOGIC, D., COLUCCI-GUYON, E., WANG, N., MANIOTIS, A., INGBER, D., MERCKLING, A., LANGA, F., AUMAILLEY, M., DELOUVEE, A., KOTELIANSKY, V., BABINET, C. & KRIEG, T. 1998. Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. *J Cell Sci,* 111 (Pt 13)**,** 1897-907.

EHRHARDT, C., COLLNOT, E. M., BALDES, C., BECKER, U., LAUE, M., KIM, K. J. & LEHR, C. M. 2006. Towards an *in vitro* model of cystic fibrosis small airway epithelium: characterisation of the human bronchial epithelial cell line CFBE41o. *Cell Tissue Res,* 323**,** 405-15.

EICKELBERG, O., KOHLER, E., REICHENBERGER, F., BERTSCHIN, S., WOODTLI, T., ERNE, P., PERRUCHOUD, A. P. & ROTH, M. 1999. Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am J Physiol,* 276**,** L814-24.

ELBORN, J. S. 1999. Treatment of Staphylococcus aureus in cystic fibrosis. *Thorax,* 54**,** 377-8.

EPELMAN, S., BRUNO, T. F., NEELY, G. G., WOODS, D. E. & MODY, C. H. 2000. Pseudomonas aeruginosa exoenzyme S induces transcriptional expression of proinflammatory cytokines and chemokines. *Infection and immunity,* 68**,** 4811-4.

ERICKSON, D. L., ENDERSBY, R., KIRKHAM, A., STUBER, K., VOLLMAN, D. D., RABIN, H. R., MITCHELL, I. & STOREY, D. G. 2002. Pseudomonas aeruginosa quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infection and immunity,* 70**,** 1783-90.

ERIKSSON, J. E., DECHAT, T., GRIN, B., HELFAND, B., MENDEZ, M., PALLARI, H. M. & GOLDMAN, R. D. 2009. Introducing intermediate filaments: from discovery to disease. *The Journal of clinical investigation,* 119**,** 1763-71.

ERNST, R. K., MOSKOWITZ, S. M., EMERSON, J. C., KRAIG, G. M., ADAMS, K. N., HARVEY, M. D., RAMSEY, B., SPEERT, D. P., BURNS, J. L. & MILLER, S. I. 2007. Unique lipid a modifications in Pseudomonas aeruginosa isolated from the airways of patients with cystic fibrosis. *The Journal of infectious diseases,* 196**,** 1088-92.

ESSMANN, F., BANTEL, H., TOTZKE, G., ENGELS, I. H., SINHA, B., SCHULZE-OSTHOFF, K. & JANICKE, R. U. 2003. Staphylococcus aureus alpha-toxin-induced cell death: predominant

necrosis despite apoptotic caspase activation. *Cell death and differentiation,* 10**,** 1260-72.

EVANS, M. J., GUHA, S. C., COX, R. A. & MOLLER, P. C. 1993. Attenuated fibroblast sheath around the basement membrane zone in the trachea. *American journal of respiratory cell and molecular biology,* 8**,** 188-92.

EVANS, M. J., VAN WINKLE, L. S., FANUCCHI, M. V. & PLOPPER, C. G. 1999. The attenuated fibroblast sheath of the respiratory tract epithelial-mesenchymal trophic unit. *American journal of respiratory cell and molecular biology,* 21**,** 655-7.

FANG, K., ZHAO, H., SUN, C., LAM, C. M., CHANG, S., ZHANG, K., PANDA, G., GODINHO, M., MARTINS DOS SANTOS, V. A. & WANG, J. 2011. Exploring the metabolic network of the epidemic pathogen Burkholderia cenocepacia J2315 via genome-scale reconstruction. *BMC systems biology,* 5**,** 83.

FARQUHAR, M. G. & PALADE, G. E. 1963. Junctional complexes in various epithelia. *The Journal of cell biology,* 17**,** 375-412.

FIEDLER, M. A., KAETZEL, C. S. & DAVIS, P. B. 1991. Sustained production of secretory component by human tracheal epithelial cells in primary culture. *Am J Physiol,* 261**,** L255-61.

FINK, J., STEER, J. H., JOYCE, D. A., MCWILLIAM, A. S. & STEWART, G. A. 2003. Pro-inflammatory effects of Burkholderia cepacia on cystic fibrosis respiratory epithelium. *FEMS immunology and medical microbiology,* 38**,** 273-82.

FIROVED, A. M., ORNATOWSKI, W. & DERETIC, V. 2004. Microarray analysis reveals induction of lipoprotein genes in mucoid Pseudomonas aeruginosa: implications for inflammation in cystic fibrosis. *Infect Immun,* 72**,** 5012-8.

FLEISZIG, S. M., EVANS, D. J., DO, N., VALLAS, V., SHIN, S. & MOSTOV, K. E. 1997. Epithelial cell polarity affects susceptibility to Pseudomonas aeruginosa invasion and cytotoxicity. *Infection and immunity,* 65**,** 2861-7.

FLOREA, B. I., CASSARA, M. L., JUNGINGER, H. E. & BORCHARD, G. 2003. Drug transport and metabolism characteristics of the human airway epithelial cell line Calu-3. *J Control Release,* 87**,** 131-8.

FLOTTE, T. R., AFIONE, S. A., SOLOW, R., DRUMM, M. L., MARKAKIS, D., GUGGINO, W. B., ZEITLIN, P. L. & CARTER, B. J. 1993. Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. *The Journal of biological chemistry,* 268**,** 3781-90.

FLOTTE, T. R., SOLOW, R., OWENS, R. A., AFIONE, S., ZEITLIN, P. L. & CARTER, B. J. 1992. Gene expression from adeno-associated virus vectors in airway epithelial cells. *American journal of respiratory cell and molecular biology,* 7**,** 349-56.

FOSTER, K. A., AVERY, M. L., YAZDANIAN, M. & AUDUS, K. L. 2000. Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. *International journal of pharmaceutics,* 208**,** 1-11.

FOURNIER, B. & PHILPOTT, D. J. 2005. Recognition of Staphylococcus aureus by the innate immune system. *Clinical microbiology reviews,* 18**,** 521-40.

FRANZDOTTIR, S. R., AXELSSON, I. T., ARASON, A. J., BALDURSSON, O., GUDJONSSON, T. & MAGNUSSON, M. K. 2010. Airway branching morphogenesis in three dimensional culture. *Respiratory research,* 11**,** 162.

GAIL, D. B. & LENFANT, C. J. 1983. Cells of the lung: biology and clinical implications. *The American review of respiratory disease,* 127**,** 366-87.

GALANOS, C. & FREUDENBERG, M. A. 1993. Bacterial endotoxins: biological properties and mechanisms of action. *Mediators of inflammation,* 2**,** S11-6.

GAO, B., WANG, Y. & TSAN, M. F. 2006. The heat sensitivity of cytokine-inducing effect of lipopolysaccharide. *Journal of leukocyte biology,* 80**,** 359-66.

GERAGHTY, P., ROGAN, M. P., GREENE, C. M., BOXIO, R. M., POIRIERT, T., O'MAHONY, M., BELAAOUAJ, A., O'NEILL, S. J., TAGGART, C. C. & MCELVANEY, N. G. 2007. Neutrophil elastase up-regulates cathepsin B and matrix metalloprotease-2 expression. *Journal of immunology,* 178**,** 5871-8.

GIBSON, L. E. & COOKE, R. E. 1959. A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. *Pediatrics,* 23**,** 545-9.

GIBSON, R. L., RETSCH-BOGART, G. Z., OERMANN, C., MILLA, C., PILEWSKI, J., DAINES, C., AHRENS, R., LEON, K., COHEN, M., MCNAMARA, S., CALLAHAN, T. L., MARKUS, R. & BURNS, J. L. 2006. Microbiology, safety, and pharmacokinetics of aztreonam lysinate for inhalation in patients with cystic fibrosis. *Pediatric pulmonology,* 41**,** 656-65.

GIROD, S., GALABERT, C., LECUIRE, A., ZAHM, J. M. & PUCHELLE, E. 1992. Phospholipid composition and surface-active properties of tracheobronchial secretions from patients with cystic fibrosis and chronic obstructive pulmonary diseases. *Pediatric pulmonology,* 13**,** 22-7.

GOLDBERG, J. B. & PIER, G. B. 2000. The role of the CFTR in susceptibility to Pseudomonas aeruginosa infections in cystic fibrosis. *Trends in microbiology,* 8**,** 514-20.

GOMEZ, M. I., LEE, A., REDDY, B., MUIR, A., SOONG, G., PITT, A., CHEUNG, A. & PRINCE, A. 2004. Staphylococcus aureus protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nature medicine,* 10**,** 842-8.

GOVAN, J. R., BROWN, P. H., MADDISON, J., DOHERTY, C. J., NELSON, J. W., DODD, M., GREENING, A. P. & WEBB, A. K. 1993. Evidence for transmission of Pseudomonas cepacia by social contact in cystic fibrosis. *Lancet,* 342**,** 15-9.

GRAINGER, C., GREENWELL, L., LOCKLEY, D., MARTIN, G. & FORBES, B. 2006. Culture of Calu-3 Cells at the Air Interface Provides a Representative Model of the Airway Epithelial Barrier. *Pharmaceutical Research,* 23**,** 1482-1490.

GREEN, K. J. & JONES, J. C. 1996. Desmosomes and hemidesmosomes: structure and function of molecular components. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 10**,** 871-81.

GREENE, C. M., CARROLL, T. P., SMITH, S. G., TAGGART, C. C., DEVANEY, J., GRIFFIN, S., O'NEILL S, J. & MCELVANEY, N. G. 2005a. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *Journal of immunology,* 174**,** 1638-46.

GREENE, C. M., CARROLL, T. P., SMITH, S. G., TAGGART, C. C., DEVANEY, J., GRIFFIN, S., O'NEILL S, J. & MCELVANEY, N. G. 2005b. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol,* 174**,** 1638-46.

GREENLEE, K. J., WERB, Z. & KHERADMAND, F. 2007. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiological reviews,* 87**,** 69-98.

GROBSTEIN, C. 1967. Mechanisms of organogenetic tissue interaction. *National Cancer Institute monograph,* 26**,** 279-99.

GROEGER, S., DOMAN, E., CHAKRABORTY, T. & MEYLE, J. 2010. Effects of Porphyromonas gingivalis infection on human gingival epithelial barrier function *in vitro*. *European journal of oral sciences,* 118**,** 582-9.

GRUENERT, D. C., BASBAUM, C. B., WELSH, M. J., LI, M., FINKBEINER, W. E. & NADEL, J. A. 1988. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proceedings of the National Academy of Sciences of the United States of America,* 85**,** 5951-5.

GRUENERT, D. C., FINKBEINER, W. E. & WIDDICOMBE, J. H. 1995. Culture and transformation of human airway epithelial cells. *Am J Physiol,* 268**,** L347-60.

GUILLOT, L., MEDJANE, S., LE-BARILLEC, K., BALLOY, V., DANEL, C., CHIGNARD, M. & SI-TAHAR, M. 2004. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem,* 279**,** 2712-8.

GUYONNET DUPERAT, V., AUDIE, J. P., DEBAILLEUL, V., LAINE, A., BUISINE, M. P., GALIEGUE-ZOUITINA, S., PIGNY, P., DEGAND, P., AUBERT, J. P. & PORCHET, N. 1995. Characterization of the human mucin gene MUC5AC: a consensus cysteine-rich domain for 11p15 mucin genes? *The Biochemical journal,* 305 (Pt 1)**,** 211-9.

HAILMAN, E., LICHENSTEIN, H. S., WURFEL, M. M., MILLER, D. S., JOHNSON, D. A., KELLEY, M., BUSSE, L. A., ZUKOWSKI, M. M. & WRIGHT, S. D. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *The Journal of experimental medicine,* 179**,** 269-77.

HAUBER, H. P., TULIC, M. K., TSICOPOULOS, A., WALLAERT, B., OLIVENSTEIN, R., DAIGNEAULT, P. & HAMID, Q. 2005. Toll-like receptors 4 and 2 expression in the bronchial mucosa of patients with cystic fibrosis. *Can Respir J,* 12**,** 13-8.

HECKMAN, C. A. 1983. Organ and species specificity of epithelial growth. *In vitro,* 19**,** 31-40.

HEECKEREN, A., WALENGA, R., KONSTAN, M. W., BONFIELD, T., DAVIS, P. B. & FERKOL, T. 1997. Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with Pseudomonas aeruginosa. *The Journal of clinical investigation,* 100**,** 2810-5.

HINENOYA, N., NAITO, I., MOMOTA, R., SADO, Y., KUMAGISHI, K., NINOMIYA, Y. & OHTSUKA, A. 2008. Type IV collagen alpha chains of the basement membrane in the rat bronchioalveolar transitional segment. *Arch Histol Cytol,* 71**,** 185-94.

HOLGATE, S. T., DAVIES, D. E., LACKIE, P. M., WILSON, S. J., PUDDICOMBE, S. M. & LORDAN, J. L. 2000. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *The Journal of allergy and clinical immunology,* 105**,** 193-204.

HORNEF, M. W., FRISAN, T., VANDEWALLE, A., NORMARK, S. & RICHTER-DAHLFORS, A. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *The Journal of experimental medicine,* 195**,** 559-70.

HOUTMEYERS, E., GOSSELINK, R., GAYAN-RAMIREZ, G. & DECRAMER, M. 1999. Regulation of mucociliary clearance in health and disease. *Eur Respir J,* 13**,** 1177-88.

HOWAT, W. J., HOLMES, J. A., HOLGATE, S. T. & LACKIE, P. M. 2001. Basement membrane pores in human bronchial epithelium: a conduit for infiltrating cells? *The American journal of pathology,* 158**,** 673-80.

HYDE, S. C., SOUTHERN, K. W., GILEADI, U., FITZJOHN, E. M., MOFFORD, K. A., WADDELL, B. E., GOOI, H. C., GODDARD, C. A., HANNAVY, K., SMYTH, S. E., EGAN, J. J., SORGI, F. L., HUANG, L., CUTHBERT, A. W., EVANS, M. J., COLLEDGE, W. H., HIGGINS, C. F., WEBB, A. K. & GILL, D. R. 2000. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene therapy,* 7**,** 1156-65.

ISLES, A., MACLUSKY, I., COREY, M., GOLD, R., PROBER, C., FLEMING, P. & LEVISON, H. 1984. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. *The Journal of pediatrics,* 104**,** 206-10.

JALILI, R. B., REZAKHANLOU, A. M., HOSSEINI-TABATABAEI, A., AO, Z., WARNOCK, G. L. & GHAHARY, A. 2010. Fibroblast populated collagen matrix promotes islet survival and reduces the number of islets required for diabetes reversal. *Journal of cellular physiology*.

JANARDHAN, K. S., MCISAAC, M., FOWLIE, J., SHRIVASTAV, A., CALDWELL, S., SHARMA, R. K. & SINGH, B. 2006. Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation. *Histology and histopathology,* 21**,** 687-96.

JEFFERY, P. K. & LI, D. 1997. Airway mucosa: secretory cells, mucus and mucin genes. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology,* 10**,** 1655-62.

JEFFERY, P. K. & REID, L. 1975. New observations of rat airway epithelium: a quantitative and electron microscopic study. *Journal of anatomy,* 120**,** 295-320.

JOHN, G., YILDIRIM, A. O., RUBIN, B. K., GRUENERT, D. C. & HENKE, M. O. 2010. TLR-4-mediated innate immunity is reduced in cystic fibrosis airway cells. *Am J Respir Cell Mol Biol,* 42**,** 424-31.

JOSEPH, T., LOOK, D. & FERKOL, T. 2005. NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa. *Am J Physiol Lung Cell Mol Physiol,* 288**,** L471-9.

KADLER, K. E., BALDOCK, C., BELLA, J. & BOOT-HANDFORD, R. P. 2007. Collagens at a glance. *J Cell Sci,* 120**,** 1955-8.

KAHL, B. C. 2010. Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease. *International journal of medical microbiology : IJMM,* 300**,** 514-9.

KAHL, B. C., GOULIAN, M., VAN WAMEL, W., HERRMANN, M., SIMON, S. M., KAPLAN, G., PETERS, G. & CHEUNG, A. L. 2000. Staphylococcus aureus RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. *Infection and immunity,* 68**,** 5385-92.

KAVVADA, K. M., MURRAY, J. G., MOORE, V. A., COOMBES, A. G. & HANSON, P. J. 2005. A collagen IV matrix is required for guinea pig gastric epithelial cell monolayers to provide an optimal model of the stomach surface for biopharmaceutical screening. *Journal of biomolecular screening,* 10**,** 495-507.

KAZA, S. K., MCCLEAN, S. & CALLAGHAN, M. 2011. IL-8 released from human lung epithelial cells induced by cystic fibrosis pathogens Burkholderia cepacia complex affects the growth and intracellular survival of bacteria. *International Journal of Medical Microbiology,* 301**,** 26-33.

KELLEY, J., FABISIAK, J. P., HAWES, K. & ABSHER, M. 1991. Cytokine signaling in lung: transforming growth factor-beta secretion by lung fibroblasts. *Am J Physiol,* 260**,** L123-8.

KHAN, T. Z., WAGENER, J. S., BOST, T., MARTINEZ, J., ACCURSO, F. J. & RICHES, D. W. 1995. Early pulmonary inflammation in infants with cystic fibrosis. *American journal of respiratory and critical care medicine,* 151**,** 1075-82.

KHOSHNOODI, J., PEDCHENKO, V. & HUDSON, B. G. 2008. Mammalian collagen IV. *Microsc Res Tech,* 71**,** 357-70.

KIKUCHI, T., SHIVELY, J. D., FOLEY, J. S., DRAZEN, J. M. & TSCHUMPERLIN, D. J. 2004. Differentiation-dependent responsiveness of bronchial epithelial cells to IL-4/13 stimulation. *American journal of physiology. Lung cellular and molecular physiology,* 287**,** L119-26.

KIM, J. I., LEE, C. J., JIN, M. S., LEE, C. H., PAIK, S. G., LEE, H. & LEE, J. O. 2005a. Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *J Biol Chem,* 280**,** 11347-51.

KIM, J. Y., SAJJAN, U. S., KRASAN, G. P. & LIPUMA, J. J. 2005b. Disruption of tight junctions during traversal of the respiratory epithelium by Burkholderia cenocepacia. *Infection and immunity,* 73**,** 7107-12.

KIM, K. C., MCCRACKEN, K., LEE, B. C., SHIN, C. Y., JO, M. J., LEE, C. J. & KO, K. H. 1997. Airway goblet cell mucin: its structure and regulation of secretion. *Eur Respir J,* 10**,** 2644-9.

KIPNIS, E., SAWA, T. & WIENER-KRONISH, J. 2006. Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Medecine et maladies infectieuses,* 36**,** 78-91.

KIRKHAM, S., SHEEHAN, J. K., KNIGHT, D., RICHARDSON, P. S. & THORNTON, D. J. 2002. Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *The Biochemical journal,* 361**,** 537-46.

KITA, E., SAWAKI, M., OKU, D., HAMURO, A., MIKASA, K., KONISHI, M., EMOTO, M., TAKEUCHI, S., NARITA, N. & KASHIBA, S. 1991. Suppression of virulence factors of Pseudomonas aeruginosa by erythromycin. *The Journal of antimicrobial chemotherapy,* 27**,** 273-84.

KLINGER, J. D., CASH, H. A., WOOD, R. E. & MILER, J. J. 1983. Protective immunization against chronic Pseudomonas aeruginosa pulmonary infection in rats. *Infection and immunity,* 39**,** 1377-84.

KNIGHT, D. 2001. Epithelium-fibroblast interactions in response to airway inflammation. *Immunol Cell Biol,* 79**,** 160-4.

KNIGHT, D. A. & HOLGATE, S. T. 2003. The airway epithelium: structural and functional properties in health and disease. *Respirology,* 8**,** 432-46.

KNOWLES, M. R. & BOUCHER, R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest,* 109**,** 571-7.

KONSTAN, M. W., DAVIS, P. B., WAGENER, J. S., HILLIARD, K. A., STERN, R. C., MILGRAM, L. J., KOWALCZYK, T. H., HYATT, S. L., FINK, T. L., GEDEON, C. R., OETTE, S. M., PAYNE, J. M., MUHAMMAD, O., ZIADY, A. G., MOEN, R. C. & COOPER, M. J. 2004. Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. *Human gene therapy,* 15**,** 1255-69.

KONSTAN, M. W., HILLIARD, K. A., NORVELL, T. M. & BERGER, M. 1994. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *American journal of respiratory and critical care medicine,* 150**,** 448-54.

KOUNNAS, M. Z., MORRIS, R. E., THOMPSON, M. R., FITZGERALD, D. J., STRICKLAND, D. K. & SAELINGER, C. B. 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *The Journal of biological chemistry,* 267**,** 12420-3.

KOYAMA, S., SATO, E., NOMURA, H., KUBO, K., MIURA, M., YAMASHITA, T., NAGAI, S. & IZUMI, T. 2000. The potential of various lipopolysaccharides to release IL-8 and G-CSF. *American journal of physiology. Lung cellular and molecular physiology,* 278**,** L658-66.

KREDA, S. M., OKADA, S. F., VAN HEUSDEN, C. A., O'NEAL, W., GABRIEL, S., ABDULLAH, L., DAVIS, C. W., BOUCHER, R. C. & LAZAROWSKI, E. R. 2007. Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol,* 584**,** 245-59.

KRIVAN, H. C., ROBERTS, D. D. & GINSBURG, V. 1988. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. *Proceedings of the National Academy of Sciences of the United States of America,* 85**,** 6157-61.

KUBE, D., SONTICH, U., FLETCHER, D. & DAVIS, P. B. 2001. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *American journal of physiology. Lung cellular and molecular physiology,* 280**,** L493-502.

KUKAVICA-IBRULJ, I. & LEVESQUE, R. C. 2008. Animal models of chronic lung infection with Pseudomonas aeruginosa: useful tools for cystic fibrosis studies. *Lab Anim,* 42**,** 389-412.

LAU, G. W., HASSETT, D. J. & BRITIGAN, B. E. 2005. Modulation of lung epithelial functions by Pseudomonas aeruginosa. *Trends in microbiology,* 13**,** 389-97.

LEBLEU, V. S., MACDONALD, B. & KALLURI, R. 2007. Structure and function of basement membranes. *Exp Biol Med (Maywood),* 232**,** 1121-9.

LECHNER, J. F., HAUGEN, A., MCCLENDON, I. A. & PETTIS, E. W. 1982. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. *In vitro,* 18**,** 633-42.

LEE, J. & KALETUNC, G. 2002. Evaluation of the heat inactivation of Escherichia coli and Lactobacillus plantarum by differential scanning calorimetry. *Applied and environmental microbiology,* 68**,** 5379-86.

LIEBER, M., SMITH, B., SZAKAL, A., NELSON-REES, W. & TODARO, G. 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *International journal of cancer. Journal international du cancer,* 17**,** 62-70.

LIPECKA, J., NOREZ, C., BENSALEM, N., BAUDOUIN-LEGROS, M., PLANELLES, G., BECQ, F., EDELMAN, A. & DAVEZAC, N. 2006. Rescue of DeltaF508-CFTR (cystic fibrosis transmembrane conductance regulator) by curcumin: involvement of the keratin 18 network. *The Journal of pharmacology and experimental therapeutics,* 317**,** 500-5.

LIPUMA, J. J. 2003. Burkholderia and emerging pathogens in cystic fibrosis. *Seminars in respiratory and critical care medicine,* 24**,** 681-92.

LIVRAGHI, A. & RANDELL, S. H. 2007. Cystic fibrosis and other respiratory diseases of impaired mucus clearance. *Toxicol Pathol,* 35**,** 116-29.

MAHENTHIRALINGAM, E., CAMPBELL, M. E. & SPEERT, D. P. 1994. Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis. *Infection and immunity,* 62**,** 596-605.

MAHENTHIRALINGAM, E., URBAN, T. A. & GOLDBERG, J. B. 2005. The multifarious, multireplicon Burkholderia cepacia complex. *Nature reviews. Microbiology,* 3**,** 144-56.

MALL, M., GRUBB, B. R., HARKEMA, J. R., O'NEAL, W. K. & BOUCHER, R. C. 2004. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med,* 10**,** 487-93.

MALL, M. A. 2008. Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *J Aerosol Med Pulm Drug Deliv,* 21**,** 13-24.

MARTIN, D. W. & MOHR, C. D. 2000. Invasion and intracellular survival of Burkholderia cepacia. *Infection and immunity,* 68**,** 24-9.

MARTIN, D. W., SCHURR, M. J., MUDD, M. H., GOVAN, J. R., HOLLOWAY, B. W. & DERETIC, V. 1993. Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 8377-81.

MATHIA, N. R., TIMOSZYK, J., STETSKO, P. I., MEGILL, J. R., SMITH, R. L. & WALL, D. A. 2002. Permeability characteristics of calu-3 human bronchial epithelial cells: *in vitro-in vivo* correlation to predict lung absorption in rats. *J Drug Target,* 10**,** 31-40.

MATSUI, H., VERGHESE, M. W., KESIMER, M., SCHWAB, U. E., RANDELL, S. H., SHEEHAN, J. K., GRUBB, B. R. & BOUCHER, R. C. 2005. Reduced three-dimensional motility in dehydrated airway mucus prevents neutrophil capture and killing bacteria on airway epithelial surfaces. *Journal of immunology,* 175**,** 1090-9.

MCANULTY, R. J., CHAMBERS, R. C. & LAURENT, G. J. 1995. Regulation of fibroblast procollagen production. Transforming growth factor-beta 1 induces prostaglandin E2 but not procollagen synthesis via a pertussis toxin-sensitive G-protein. *The Biochemical journal,* 307 (Pt 1)**,** 63-8.

MCCLEAN, S. & CALLAGHAN, M. 2009. Burkholderia cepacia complex: epithelial cell-pathogen confrontations and potential for therapeutic intervention. *Journal of medical microbiology,* 58**,** 1-12.

MCCOY, K. S., QUITTNER, A. L., OERMANN, C. M., GIBSON, R. L., RETSCH-BOGART, G. Z. & MONTGOMERY, A. B. 2008. Inhaled aztreonam lysine for chronic airway Pseudomonas aeruginosa in cystic fibrosis. *American journal of respiratory and critical care medicine,* 178**,** 921-8.

MCKEON, S. A., NGUYEN, D. T., VITERI, D. F., ZLOSNIK, J. E. & SOKOL, P. A. 2011. Functional quorum sensing systems are maintained during chronic Burkholderia cepacia complex infections in patients with cystic fibrosis. *The Journal of infectious diseases,* 203**,** 383-92.

MENZIES, B. E. & KENOYER, A. 2005. Staphylococcus aureus infection of epidermal keratinocytes promotes expression of innate antimicrobial peptides. *Infection and immunity,* 73**,** 5241-4.

MILLS, P. R., DAVIES, R. J. & DEVALIA, J. L. 1999. Airway epithelial cells, cytokines, and pollutants. *Am J Respir Crit Care Med,* 160**,** S38-43.

MINOO, P. & KING, R. J. 1994. Epithelial-mesenchymal interactions in lung development. *Annual review of physiology,* 56**,** 13-45.

MITHAL, A. & EMERY, J. L. 1976. Squamous metaplasia of the tracheal epithelium in children. *Thorax,* 31**,** 167-71.

MIYAZAKI, K., HATTORI, Y., UMENISHI, F., YASUMITSU, H. & UMEDA, M. 1990. Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. *Cancer Res,* 50**,** 7758-64.

MOGHAL, N. & NEEL, B. G. 1998. Integration of growth factor, extracellular matrix, and retinoid signals during bronchial epithelial cell differentiation. *Molecular and cellular biology,* 18**,** 6666-78.

MOLL, R., DIVO, M. & LANGBEIN, L. 2008. The human keratins: biology and pathology. *Histochemistry and cell biology,* 129**,** 705-33.

MOLL, R., FRANKE, W. W., SCHILLER, D. L., GEIGER, B. & KREPLER, R. 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell,* 31**,** 11-24.

MORRIS, A., STEINBERG, M. L. & DEFENDI, V. 1985. Keratin gene expression in simian virus 40-transformed human keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America,* 82**,** 8498-502.

MOSS, R. B., MILLA, C., COLOMBO, J., ACCURSO, F., ZEITLIN, P. L., CLANCY, J. P., SPENCER, L. T., PILEWSKI, J., WALTZ, D. A., DORKIN, H. L., FERKOL, T., PIAN, M., RAMSEY, B., CARTER, B. J., MARTIN, D. B. & HEALD, A. E. 2007. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Human gene therapy,* 18**,** 726-32.

MOSS, R. B., RODMAN, D., SPENCER, L. T., AITKEN, M. L., ZEITLIN, P. L., WALTZ, D., MILLA, C., BRODY, A. S., CLANCY, J. P., RAMSEY, B., HAMBLETT, N. & HEALD, A. E. 2004. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest,* 125**,** 509-21.

MOURA, J. A., CRISTINA DE ASSIS, M., VENTURA, G. C., SALIBA, A. M., GONZAGA, L., JR., SI-TAHAR, M., MARQUES EDE, A. & PLOTKOWSKI, M. C. 2008. Differential interaction of bacterial species from the Burkholderia cepacia complex with human airway epithelial cells. *Microbes and infection / Institut Pasteur,* 10**,** 52-9.

MUIR, A., SOONG, G., SOKOL, S., REDDY, B., GOMEZ, M. I., VAN HEECKEREN, A. & PRINCE, A. 2004. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol,* 30**,** 777-83.

MUTSAERS, S. E., BISHOP, J. E., MCGROUTHER, G. & LAURENT, G. J. 1997. Mechanisms of tissue repair: from wound healing to fibrosis. *The International Journal of Biochemistry & Cell Biology,* 29**,** 5-17.

MYERBURG, M. M., LATOCHE, J. D., MCKENNA, E. E., STABILE, L. P., SIEGFRIED, J. S., FEGHALI-BOSTWICK, C. A. & PILEWSKI, J. M. 2007. Hepatocyte growth factor and other fibroblast secretions modulate the phenotype of human bronchial epithelial cells. *American journal of physiology. Lung cellular and molecular physiology,* 292**,** L1352-60.

NGUYEN, D., EMOND, M. J., MAYER-HAMBLETT, N., SAIMAN, L., MARSHALL, B. C. & BURNS, J. L. 2007. Clinical response to azithromycin in cystic fibrosis correlates with *in vitro* effects on Pseudomonas aeruginosa phenotypes. *Pediatric pulmonology,* 42**,** 533-41.

NICOLETTI, I., MIGLIORATI, G., PAGLIACCI, M. C., GRIGNANI, F. & RICCARDI, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods,* 139**,** 271-9.

NILSSON, H. E., DRAGOMIR, A., LAZOROVA, L., JOHANNESSON, M. & ROOMANS, G. M. 2010. CFTR and tight junctions in cultured bronchial epithelial cells. *Experimental and Molecular Pathology,* 88**,** 118-127.

NORMARK, B. H., NORMARK, S. & NORRBY-TEGLUND, A. 2004. Staphylococcal protein A inflames the lungs. *Nature medicine,* 10**,** 780-1.

O'CONNOR, C. M. & FITZGERALD, M. X. 1994. Matrix metalloproteases and lung disease. *Thorax,* 49**,** 602-9.

O'GRADY, M. 2007. Toll Receptor Regulation of Innate Immune Function in Human Airway Eoithelial (Calu-3) Cells. *Meeting abstract supplement*.

ORIOLO, A. S., WALD, F. A., RAMSAUER, V. P. & SALAS, P. J. 2007. Intermediate filaments: a role in epithelial polarity. *Experimental cell research,* 313**,** 2255-64.

PACE, J. L., ROSSI, H. A., ESPOSITO, V. M., FREY, S. M., TUCKER, K. D. & WALKER, R. I. 1998. Inactivated whole-cell bacterial vaccines: current status and novel strategies. *Vaccine,* 16**,** 1563-74.

PALADINO, P., CUMMINGS, D. T., NOYCE, R. S. & MOSSMAN, K. L. 2006. The IFN-independent response to virus particle entry provides a first line of antiviral defense that is independent of TLRs and retinoic acid-inducible gene I. *Journal of immunology,* 177**,** 8008-16.

PALFREYMAN, R. W., WATSON, M. L., EDEN, C. & SMITH, A. W. 1997. Induction of biologically active interleukin-8 from lung epithelial cells by Burkholderia (Pseudomonas) cepacia products. *Infection and immunity,* 65**,** 617-22.

PALMER, K. L., MASHBURN, L. M., SINGH, P. K. & WHITELEY, M. 2005. Cystic fibrosis sputum supports growth and cues key aspects of Pseudomonas aeruginosa physiology. *Journal of bacteriology,* 187**,** 5267-77.

PARKER, J., SARLANG, S., THAVAGNANAM, S., WILLIAMSON, G., O'DONOGHUE, D., VILLENAVE, R., POWER, U., SHIELDS, M., HEANEY, L. & SKIBINSKI, G. 2010. A 3-D well-differentiated model of pediatric bronchial epithelium demonstrates unstimulated morphological differences between asthmatic and nonasthmatic cells. *Pediatr Res,* 67**,** 17-22.

PEREZ, A., ISSLER, A. C., COTTON, C. U., KELLEY, T. J., VERKMAN, A. S. & DAVIS, P. B. 2007. CFTR inhibition mimics the cystic fibrosis inflammatory profile. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 292**,** L383-L395.

PIER, G. B. 2002. CFTR mutations and host susceptibility to Pseudomonas aeruginosa lung infection. *Current opinion in microbiology,* 5**,** 81-6.

PIER, G. B., GROUT, M., ZAIDI, T. S., OLSEN, J. C., JOHNSON, L. G., YANKASKAS, J. R. & GOLDBERG, J. B. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science,* 271**,** 64-7.

PIER, G. B., MELULENI, G. & NEUGER, E. 1992. A murine model of chronic mucosal colonization by Pseudomonas aeruginosa. *Infection and immunity,* 60**,** 4768-76.

PLOSKER, G. L. 2010. Aztreonam lysine for inhalation solution: in cystic fibrosis. *Drugs,* 70**,** 1843-55.

POXTON, I. R. 1995. Antibodies to lipopolysaccharide. *Journal of immunological methods,* 186**,** 1-15.

PURKIS, P. E., STEEL, J. B., MACKENZIE, I. C., NATHRATH, W. B., LEIGH, I. M. & LANE, E. B. 1990. Antibody markers of basal cells in complex epithelia. *Journal of cell science,* 97 (Pt 1)**,** 39-50.

RAJAN, S., CACALANO, G., BRYAN, R., RATNER, A. J., SONTICH, C. U., VAN HEERCKEREN, A., DAVIS, P. & PRINCE, A. 2000. Pseudomonas aeruginosa induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. *American journal of respiratory cell and molecular biology,* 23**,** 304-12.

RAMESH BABU, P. B., CHIDEKEL, A., UTIDJIAN, L. & SHAFFER, T. H. 2004. Regulation of apical surface fluid and protein secretion in human airway epithelial cell line Calu-3. *Biochem Biophys Res Commun,* 319**,** 1132-7.

RAMPHAL, R., CARNOY, C., FIEVRE, S., MICHALSKI, J. C., HOUDRET, N., LAMBLIN, G., STRECKER, G. & ROUSSEL, P. 1991. Pseudomonas aeruginosa recognizes carbohydrate chains containing type 1 (Gal beta 1-3GlcNAc) or type 2 (Gal beta 1-4GlcNAc) disaccharide units. *Infection and immunity,* 59**,** 700-4.

RANDELL, S. H., FULCHER, M. L., O'NEAL, W. & OLSEN, J. C. 2011. Primary epithelial cell models for cystic fibrosis research. *Methods in molecular biology,* 742**,** 285-310.

RAOUST, E., BALLOY, V., GARCIA-VERDUGO, I., TOUQUI, L., RAMPHAL, R. & CHIGNARD, M. 2009. Pseudomonas aeruginosa LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. *PLoS One,* 4**,** e7259.

RATJEN, F. & DORING, G. 2003. Cystic fibrosis. *Lancet,* 361**,** 681-9.

RATJEN, F., HARTOG, C. M., PAUL, K., WERMELT, J. & BRAUN, J. 2002. Matrix metalloproteases in BAL fluid of patients with cystic fibrosis and their modulation by treatment with dornase alpha. *Thorax,* 57**,** 930-4.

RATJEN, F. A. 2009. Cystic fibrosis: pathogenesis and future treatment strategies. *Respir Care,* 54**,** 595-605.

RATNER, A. J., BRYAN, R., WEBER, A., NGUYEN, S., BARNES, D., PITT, A., GELBER, S., CHEUNG, A. & PRINCE, A. 2001. Cystic fibrosis pathogens activate Ca2+-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J Biol Chem,* 276**,** 19267-75.

RAVIOLA, G., SAGATIES, M. J. & MILLER, C. 1987. Intercellular junctions between fibroblasts in connective tissues of the eye of macaque monkeys. A thin section and freeze fracture analysis. *Investigative ophthalmology & visual science,* 28**,** 834-41.

RAZVI, S., QUITTELL, L., SEWALL, A., QUINTON, H., MARSHALL, B. & SAIMAN, L. 2009. Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. *Chest,* 136**,** 1554-60.

REDDEL, R. R., KE, Y., GERWIN, B. I., MCMENAMIN, M. G., LECHNER, J. F., SU, R. T., BRASH, D. E., PARK, J. B., RHIM, J. S. & HARRIS, C. C. 1988. Transformation of human bronchial

epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res,* 48**,** 1904-9.

RIETSCHEL, E. T., KIRIKAE, T., SCHADE, F. U., MAMAT, U., SCHMIDT, G., LOPPNOW, H., ULMER, A. J., ZAHRINGER, U., SEYDEL, U., DI PADOVA, F. & *ET AL*. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 8**,** 217-25.

ROBINSON, C. B. & WU, R. 1993. Mucin synthesis and secretion by cultured tracheal cells: effects of collagen gel substratum thickness. *In vitro cellular & developmental biology. Animal,* 29A**,** 469-77.

ROGERS, A. V., DEWAR, A., CORRIN, B. & JEFFERY, P. K. 1993. Identification of serous-like cells in the surface epithelium of human bronchioles. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology,* 6**,** 498-504.

ROGERS, C. S., STOLTZ, D. A., MEYERHOLZ, D. K., OSTEDGAARD, L. S., ROKHLINA, T., TAFT, P. J., ROGAN, M. P., PEZZULO, A. A., KARP, P. H., ITANI, O. A., KABEL, A. C., WOHLFORD-LENANE, C. L., DAVIS, G. J., HANFLAND, R. A., SMITH, T. L., SAMUEL, M., WAX, D., MURPHY, C. N., RIEKE, A., WHITWORTH, K., UC, A., STARNER, T. D., BROGDEN, K. A., SHILYANSKY, J., MCCRAY, P. B., JR., ZABNER, J., PRATHER, R. S. & WELSH, M. J. 2008. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science,* 321**,** 1837-41.

ROLFE, M. W., KUNKEL, S. L., STANDIFORD, T. J., CHENSUE, S. W., ALLEN, R. M., EVANOFF, H. L., PHAN, S. H. & STRIETER, R. M. 1991. Pulmonary fibroblast expression of interleukin-8: a model for alveolar macrophage-derived cytokine networking. *American journal of respiratory cell and molecular biology,* 5**,** 493-501.

ROUSSEL, P., LAMBLIN, G. & DEGAND, P. 1975. Heterogeneity of the carbohydrate chains of sulfated bronchial glycoproteins isolated from a patient suffering from cystic fibrosis. *The Journal of biological chemistry,* 250**,** 2114-22.

ROVAI, L. E., HERSCHMAN, H. R. & SMITH, J. B. 1998. The murine neutrophil-chemoattractant chemokines LIX, KC, and MIP-2 have distinct induction kinetics, tissue distributions, and tissue-specific sensitivities to glucocorticoid regulation in endotoxemia. *Journal of leukocyte biology,* 64**,** 494-502.

RUIZ, F. E., CLANCY, J. P., PERRICONE, M. A., BEBOK, Z., HONG, J. S., CHENG, S. H., MEEKER, D. P., YOUNG, K. R., SCHOUMACHER, R. A., WEATHERLY, M. R., WING, L., MORRIS, J. E., SINDEL, L., ROSENBERG, M., VAN GINKEL, F. W., MCGHEE, J. R., KELLY, D., LYRENE, R. K. & SORSCHER, E. J. 2001. A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. *Human gene therapy,* 12**,** 751-61.

SACCO, O., SILVESTRI, M., SABATINI, F., SALE, R., DEFILIPPI, A.-C. & ROSSI, G. A. 2004. Epithelial cells and fibroblasts: structural repair and remodelling in the airways. *Paediatric Respiratory Reviews,* 5**,** S35-S40.

SAGE, H. 1982. Collagens of basement membranes. *J Invest Dermatol,* 79 Suppl 1**,** 51s-59s.

SAGEL, S. D., KAPSNER, R. K. & OSBERG, I. 2005. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. *Pediatr Pulmonol,* 39**,** 224-32.

SAIMAN, L., MARSHALL, B. C., MAYER-HAMBLETT, N., BURNS, J. L., QUITTNER, A. L., CIBENE, D. A., COQUILLETTE, S., FIEBERG, A. Y., ACCURSO, F. J. & CAMPBELL, P. W., 3RD 2003. Azithromycin in patients with cystic fibrosis chronically infected with Pseudomonas aeruginosa: a randomized controlled trial. *JAMA : the journal of the American Medical Association,* 290**,** 1749-56.

SAIMAN, L. & PRINCE, A. 1993. Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *The Journal of clinical investigation,* 92**,** 1875-80.

SAJJAN, S. U. & FORSTNER, J. F. 1992. Identification of the mucin-binding adhesin of Pseudomonas cepacia isolated from patients with cystic fibrosis. *Infection and immunity,* 60**,** 1434-40.

SAJJAN, U., COREY, M., HUMAR, A., TULLIS, E., CUTZ, E., ACKERLEY, C. & FORSTNER, J. 2001. Immunolocalisation of Burkholderia cepacia in the lungs of cystic fibrosis patients. *Journal of medical microbiology,* 50**,** 535-46.

SAJJAN, U., WU, Y., KENT, G. & FORSTNER, J. 2000a. Preferential adherence of cable-piliated burkholderia cepacia to respiratory epithelia of CF knockout mice and human cystic fibrosis lung explants. *Journal of medical microbiology,* 49**,** 875-85.

SAJJAN, U. S., HERSHENSON, M. B., FORSTNER, J. F. & LIPUMA, J. J. 2008. Burkholderia cenocepacia ET12 strain activates TNFR1 signalling in cystic fibrosis airway epithelial cells. *Cell Microbiol,* 10**,** 188-201.

SAJJAN, U. S., SYLVESTER, F. A. & FORSTNER, J. F. 2000b. Cable-piliated Burkholderia cepacia binds to cytokeratin 13 of epithelial cells. *Infection and immunity,* 68**,** 1787-95.

SAJJAN, U. S., YANG, J. H., HERSHENSON, M. B. & LIPUMA, J. J. 2006. Intracellular trafficking and replication of Burkholderia cenocepacia in human cystic fibrosis airway epithelial cells. *Cell Microbiol,* 8**,** 1456-66.

SALDIAS, M. S. & VALVANO, M. A. 2009. Interactions of Burkholderia cenocepacia and other Burkholderia cepacia complex bacteria with epithelial and phagocytic cells. *Microbiology,* 155**,** 2809-17.

SCHEID, P., KEMPSTER, L., GRIESENBACH, U., DAVIES, J. C., DEWAR, A., WEBER, P. P., COLLEDGE, W. H., EVANS, M. J., GEDDES, D. M. & ALTON, E. W. 2001. Inflammation in cystic fibrosis airways: relationship to increased bacterial adherence. *Eur Respir J,* 17**,** 27-35.

SCHROEDER, T. H., LEE, M. M., YACONO, P. W., CANNON, C. L., GERCEKER, A. A., GOLAN, D. E. & PIER, G. B. 2002. CFTR is a pattern recognition molecule that extracts Pseudomonas aeruginosa LPS from the outer membrane into epithelial cells and activates NF-kappa B translocation. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 6907-12.

SCHROEDER, T. H., REINIGER, N., MELULENI, G., GROUT, M., COLEMAN, F. T. & PIER, G. B. 2001a. Transgenic cystic fibrosis mice exhibit reduced early clearance of Pseudomonas aeruginosa from the respiratory tract. *Journal of immunology,* 166**,** 7410-8.

SCHROEDER, T. H., ZAIDI, T. & PIER, G. B. 2001b. Lack of adherence of clinical isolates of Pseudomonas aeruginosa to asialo-GM(1) on epithelial cells. *Infection and immunity,* 69**,** 719-29.

SCHWEIZER, J., BOWDEN, P. E., COULOMBE, P. A., LANGBEIN, L., LANE, E. B., MAGIN, T. M., MALTAIS, L., OMARY, M. B., PARRY, D. A., ROGERS, M. A. & WRIGHT, M. W. 2006. New consensus nomenclature for mammalian keratins. *The Journal of cell biology,* 174**,** 169-74.

SCHWIEBERT, L. M., ESTELL, K. & PROPST, S. M. 1999. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *The American journal of physiology,* 276**,** C700-10.

SHAN, J., HUANG, J., LIAO, J., ROBERT, R. & HANRAHAN, J. W. 2011. Anion secretion by a model epithelium: more lessons from Calu-3. *Acta physiologica,* 202**,** 523-31.

SHAW, D., POXTON, I. R. & GOVAN, J. R. 1995. Biological activity of Burkholderia (Pseudomonas) cepacia lipopolysaccharide. *FEMS immunology and medical microbiology,* 11**,** 99-106.

SHEN, B. Q., FINKBEINER, W. E., WINE, J. J., MRSNY, R. J. & WIDDICOMBE, J. H. 1994. Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion. *Am J Physiol,* 266**,** L493-501.

SHOJI, S., RICKARD, K. A., ERTL, R. F., LINDER, J. & RENNARD, S. I. 1989. Lung fibroblasts produce chemotactic factors for bronchial epithelial cells. *Am J Physiol,* 257**,** L71-9.

SHUTE, J., MARSHALL, L., BODEY, K. & BUSH, A. 2003. Growth factors in cystic fibrosis - when more is not enough. *Paediatric Respiratory Reviews,* 4**,** 120-7.

SILIPO, A., MOLINARO, A., IERANO, T., DE SOYZA, A., STURIALE, L., GAROZZO, D., ALDRIDGE, C., CORRIS, P. A., KHAN, C. M., LANZETTA, R. & PARRILLI, M. 2007. The complete structure and pro-inflammatory activity of the lipooligosaccharide of the highly epidemic and virulent gram-negative bacterium Burkholderia cenocepacia ET-12 (strain J2315). *Chemistry,* 13**,** 3501-11.

SINGER, K. H., SCEARCE, R. M., TUCK, D. T., WHICHARD, L. P., DENNING, S. M. & HAYNES, B. F. 1989. Removal of fibroblasts from human epithelial cell cultures with use of a complement fixing monoclonal antibody reactive with human fibroblasts and monocytes/macrophages. *The Journal of investigative dermatology,* 92**,** 166-70.

SINGH, P. K., PARSEK, M. R., GREENBERG, E. P. & WELSH, M. J. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature,* 417**,** 552-5.

SKIBINSKI, G., ELBORN, J. S. & ENNIS, M. 2007. Bronchial epithelial cell growth regulation in fibroblast cocultures: the role of hepatocyte growth factor. *Am J Physiol Lung Cell Mol Physiol,* 293**,** L69-76.

SMART, S. J. & CASALE, T. B. 1993. Interleukin-8-induced transcellular neutrophil migration is facilitated by endothelial and pulmonary epithelial cells. *American journal of respiratory cell and molecular biology,* 9**,** 489-95.

SMITH, E. E., BUCKLEY, D. G., WU, Z., SAENPHIMMACHAK, C., HOFFMAN, L. R., D'ARGENIO, D. A., MILLER, S. I., RAMSEY, B. W., SPEERT, D. P., MOSKOWITZ, S. M., BURNS, J. L., KAUL, R. & OLSON, M. V. 2006. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 8487-92.

SMITH, R. S., FEDYK, E. R., SPRINGER, T. A., MUKAIDA, N., IGLEWSKI, B. H. & PHIPPS, R. P. 2001. IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. *J Immunol,* 167**,** 366-74.

SNOUWAERT, J. N., BRIGMAN, K. K., LATOUR, A. M., MALOUF, N. N., BOUCHER, R. C., SMITHIES, O. & KOLLER, B. H. 1992. An animal model for cystic fibrosis made by gene targeting. *Science,* 257**,** 1083-8.

SOONG, G., MARTIN, F. J., CHUN, J., COHEN, T. S., AHN, D. S. & PRINCE, A. 2011. Staphylococcus aureus protein A mediates invasion across airway epithelial cells through activation of RhoA signaling and proteolytic activity. *The Journal of biological chemistry*.

SOONG, G., REDDY, B., SOKOL, S., ADAMO, R. & PRINCE, A. 2004. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *The Journal of clinical investigation,* 113**,** 1482-9.

STANLEY, J. R., WOODLEY, D. T., KATZ, S. I. & MARTIN, G. R. 1982. Structure and function of basement membrane. *J Invest Dermatol,* 79 Suppl 1**,** 69s-72s.

STARKE, J. R., EDWARDS, M. S., LANGSTON, C. & BAKER, C. J. 1987. A mouse model of chronic pulmonary infection with Pseudomonas aeruginosa and Pseudomonas cepacia. *Pediatric research,* 22**,** 698-702.

STEINBERG, M. L. & DEFENDI, V. 1985. Altered patterns of keratin synthesis in human epidermal keratinocytes transformed by SV40. *Journal of cellular physiology,* 123**,** 117-25.

STEVENSON, B. R., SILICIANO, J. D., MOOSEKER, M. S. & GOODENOUGH, D. A. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *The Journal of cell biology,* 103**,** 755-66.

STOLTZ, D. A., MEYERHOLZ, D. K., PEZZULO, A. A., RAMACHANDRAN, S., ROGAN, M. P., DAVIS, G. J., HANFLAND, R. A., WOHLFORD-LENANE, C., DOHRN, C. L., BARTLETT, J. A., NELSON, G. A. T., CHANG, E. H., TAFT, P. J., LUDWIG, P. S., ESTIN, M., HORNICK, E. E., LAUNSPACH, J. L., SAMUEL, M., ROKHLINA, T., KARP, P. H., OSTEDGAARD, L. S., UC, A., STARNER, T. D., HORSWILL, A. R., BROGDEN, K. A., PRATHER, R. S., RICHTER, S. S., SHILYANSKY, J., MCCRAY, P. B., JR., ZABNER, J. & WELSH, M. J. 2010. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Science translational medicine,* 2**,** 29ra31.

SUN, X., SUI, H., FISHER, J. T., YAN, Z., LIU, X., CHO, H. J., JOO, N. S., ZHANG, Y., ZHOU, W., YI, Y., KINYON, J. M., LEI-BUTTERS, D. C., GRIFFIN, M. A., NAUMANN, P., LUO, M., ASCHER, J., WANG, K., FRANA, T., WINE, J. J., MEYERHOLZ, D. K. & ENGELHARDT, J. F. 2010. Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. *The Journal of clinical investigation,* 120**,** 3149-60.

TARRAN, R. 2004. Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc,* 1**,** 42-6.

TETI, A. 1992. Regulation of cellular functions by extracellular matrix. *J Am Soc Nephrol,* 2**,** S83-7.

THAN, M. E., HENRICH, S., HUBER, R., RIES, A., MANN, K., KUHN, K., TIMPL, R., BOURENKOV, G. P., BARTUNIK, H. D. & BODE, W. 2002. The 1.9-A crystal structure of the noncollagenous (NC1) domain of human placenta collagen IV shows stabilization via a novel type of covalent Met-Lys cross-link. *Proc Natl Acad Sci U S A,* 99**,** 6607-12.

THOMAS, K. R. & CAPECCHI, M. R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell,* 51**,** 503-12.

THOMPSON, A. B., ROBBINS, R. A., ROMBERGER, D. J., SISSON, J. H., SPURZEM, J. R., TESCHLER, H. & RENNARD, S. I. 1995. Immunological functions of the pulmonary epithelium. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology,* 8**,** 127-49.

THORNTON, D. J., CARLSTEDT, I., HOWARD, M., DEVINE, P. L., PRICE, M. R. & SHEEHAN, J. K. 1996. Respiratory mucins: identification of core proteins and glycoforms. *The Biochemical journal,* 316 (Pt 3)**,** 967-75.

THORNTON, D. J. & SHEEHAN, J. K. 2004. From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc,* 1**,** 54-61.

TIMPL, R. 1989. Structure and biological activity of basement membrane proteins. *Eur J Biochem,* 180**,** 487-502.

TSAI, R. J., HO, Y. S. & CHEN, J. K. 1994. The effects of fibroblasts on the growth and differentiation of human bulbar conjunctival epithelial cells in an *in vitro* conjunctival equivalent. *Investigative ophthalmology & visual science,* 35**,** 2865-75.

ULRICH, M., HERBERT, S., BERGER, J., BELLON, G., LOUIS, D., MUNKER, G. & DORING, G. 1998. Localization of Staphylococcus aureus in infected airways of patients with cystic fibrosis and in a cell culture model of *S. aureus* adherence. *American journal of respiratory cell and molecular biology,* 19**,** 83-91.

VANDIVIER, R. W., HENSON, P. M. & DOUGLAS, I. S. 2006. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest,* 129**,** 1673-82.

VANDIVIER, R. W., RICHENS, T. R., HORSTMANN, S. A., DECATHELINEAU, A. M., GHOSH, M., REYNOLDS, S. D., XIAO, Y. Q., RICHES, D. W., PLUMB, J., VACHON, E., DOWNEY, G. P. & HENSON, P. M. 2009. Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis of apoptotic cells with proinflammatory consequences. *American journal of physiology. Lung cellular and molecular physiology,* 297**,** L677-86.

VLODAVSKY, I., LUI, G. M. & GOSPODAROWICZ, D. 1980. Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix versus plastic. *Cell,* 19**,** 607-16.

VOYNOW, J. A. & RUBIN, B. K. 2009. Mucins, mucus, and sputum. *Chest,* 135**,** 505-12.

WALTERS, R. W., GRUNST, T., BERGELSON, J. M., FINBERG, R. W., WELSH, M. J. & ZABNER, J. 1999. Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *The Journal of biological chemistry,* 274**,** 10219-26.

WAN, H., WINTON, H. L., SOELLER, C., STEWART, G. A., THOMPSON, P. J., GRUENERT, D. C., CANNELL, M. B., GARROD, D. R. & ROBINSON, C. 2000. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o. *Eur Respir J,* 15**,** 1058-68.

WANG, A., YOKOSAKI, Y., FERRANDO, R., BALMES, J. & SHEPPARD, D. 1996. Differential regulation of airway epithelial integrins by growth factors. *American journal of respiratory cell and molecular biology,* 15**,** 664-72.

WANG, N. & STAMENOVIC, D. 2002. Mechanics of vimentin intermediate filaments. *Journal of muscle research and cell motility,* 23**,** 535-40.

WELSH, M. J. & SMITH, A. E. 1995. Cystic fibrosis. *Scientific American,* 273**,** 52-9.

WESTPHAL, O., WESTPHAL, U., SOMMER, T. (ed.) 1977. *Microbiology,* Washington: Schlessinger.

WHITCUTT, M. J., ADLER, K. B. & WU, R. 1988. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In vitro cellular & developmental biology : journal of the Tissue Culture Association,* 24**,** 420-8.

WIESEL, J. M., GAMIEL, H., VLODAVSKY, I., GAY, I. & BEN-BASSAT, H. 1983. Cell attachment, growth characteristics and surface morphology of human upper-respiratory tract epithelium cultured on extracellular matrix. *European journal of clinical investigation,* 13**,** 57-63.

WINE, J. J. 1995. Cystic fibrosis: How do CFTR mutations cause cystic fibrosis? *Curr Biol,* 5**,** 1357-9.

WINE, J. J. 1999. The genesis of cystic fibrosis lung disease. *J Clin Invest,* 103**,** 309-12.

WISZNIEWSKI, L., JORNOT, L., DUDEZ, T., PAGANO, A., ROCHAT, T., LACROIX, J. S., SUTER, S. & CHANSON, M. 2006. Long-Term Cultures of Polarized Airway Epithelial Cells from Patients with Cystic Fibrosis. *Am. J. Respir. Cell Mol. Biol.,* 34**,** 39-48.

WONG, Y., SETHU, C., LOUAFI, F. & HOSSAIN, P. 2011. Lipopolysaccharide regulation of toll-like receptor-4 and matrix metalloprotease-9 in human primary corneal fibroblasts. *Investigative ophthalmology & visual science,* 52**,** 2796-803.

WU, H., SONG, Z., GIVSKOV, M., DORING, G., WORLITZSCH, D., MATHEE, K., RYGAARD, J. & HOIBY, N. 2001. Pseudomonas aeruginosa mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection. *Microbiology,* 147**,** 1105-13.

WU, R., YANKASKAS, J., CHENG, E., KNOWLES, M. R. & BOUCHER, R. 1985. Growth and differentiation of human nasal epithelial cells in culture. Serum-free, hormone-supplemented medium and proteoglycan synthesis. *The American review of respiratory disease,* 132**,** 311-20.

WU, R., ZHAO, Y. H. & CHANG, M. M. 1997. Growth and differentiation of conducting airway epithelial cells in culture. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology,* 10**,** 2398-403.

XIA, B., ROYALL, J. A., DAMERA, G., SACHDEV, G. P. & CUMMINGS, R. D. 2005. Altered O-glycosylation and sulfation of airway mucins associated with cystic fibrosis. *Glycobiology,* 15**,** 747-75.

YAMAMOTO, T., IYONAGA, K., TAKEYA, M., SAITA, N., SUGA, M., ANDO, M. & TAKAHASHI, K. 1998. Morphological alteration of cultured tracheobronchial epithelial cells is accompanied by the expression of chemokines, MCP-1 and CINC/gro, in rats. *International journal of experimental pathology,* 79**,** 81-92.

YANKASKAS, J. R., COTTON, C. U., KNOWLES, M. R., GATZY, J. T. & BOUCHER, R. C. 1985. Culture of human nasal epithelial cells on collagen matrix supports. A comparison of bioelectric properties of normal and cystic fibrosis epithelia. *The American review of respiratory disease,* 132**,** 1281-7.

YARWOOD, J. M. & SCHLIEVERT, P. M. 2003. Quorum sensing in Staphylococcus infections. *The Journal of clinical investigation,* 112**,** 1620-5.

YOON, J. H., KOO, J. S., NORFORD, D., GUZMAN, K., GRAY, T. & NETTESHEIM, P. 1999. Lysozyme expression during metaplastic squamous differentiation of retinoic acid-deficient human tracheobronchial epithelial cells. *American journal of respiratory cell and molecular biology,* 20**,** 573-81.

ZABNER, J., CHENG, S. H., MEEKER, D., LAUNSPACH, J., BALFOUR, R., PERRICONE, M. A., MORRIS, J. E., MARSHALL, J., FASBENDER, A., SMITH, A. E. & WELSH, M. J. 1997. Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia *in vivo*. *The Journal of clinical investigation,* 100**,** 1529-37.

ZABNER, J., RAMSEY, B. W., MEEKER, D. P., AITKEN, M. L., BALFOUR, R. P., GIBSON, R. L., LAUNSPACH, J., MOSCICKI, R. A., RICHARDS, S. M., STANDAERT, T. A. & *ET AL*. 1996. Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. *The Journal of clinical investigation,* 97**,** 1504-11.

ZEIHER, B. G., EICHWALD, E., ZABNER, J., SMITH, J. J., PUGA, A. P., MCCRAY, P. B., JR., CAPECCHI, M. R., WELSH, M. J. & THOMAS, K. R. 1995. A mouse model for the delta F508 allele of cystic fibrosis. *The Journal of clinical investigation,* 96**,** 2051-64.

ZEITLIN, P. L., LU, L., RHIM, J., CUTTING, G., STETTEN, G., KIEFFER, K. A., CRAIG, R. & GUGGINO, W. B. 1991a. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. *American journal of respiratory cell and molecular biology,* 4**,** 313-9.

ZEITLIN, P. L., LU, L., RHIM, J., CUTTING, G., STETTEN, G., KIEFFER, K. A., CRAIG, R. & GUGGINO, W. B. 1991b. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. *Am J Respir Cell Mol Biol,* 4**,** 313-9.

ZHANG, J., WU, L. & QU, J. M. 2011. Inhibited proliferation of human lung fibroblasts by LPS is through IL-6 and IL-8 release. *Cytokine,* 54**,** 289-95.

ZHANG, X. W., LIU, Q., WANG, Y. & THORLACIUS, H. 2001a. CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration *in vivo*. *British journal of pharmacology,* 133**,** 413-21.

ZHANG, Y., REENSTRA, W. W. & CHIDEKEL, A. 2001b. Antibacterial Activity of Apical Surface Fluid from the Human Airway Cell Line Calu-3 . Pharmacologic Alteration by Corticosteroids and beta 2-Agonists. *Am. J. Respir. Cell Mol. Biol.,* 25**,** 196-202.

ZHANG, Z., LOUBOUTIN, J. P., WEINER, D. J., GOLDBERG, J. B. & WILSON, J. M. 2005. Human airway epithelial cells sense Pseudomonas aeruginosa infection via recognition of flagellin by Toll-like receptor 5. *Infection and immunity,* 73**,** 7151-60.

ZOLLNER, T. M., RENZ, H., IGNEY, F. H. & ASADULLAH, K. 2004. Animal models of T-cell-mediated skin diseases. *BioEssays : news and reviews in molecular, cellular and developmental biology,* 26**,** 693-6.

ZUCKERMAN, J. B., ROBINSON, C. B., MCCOY, K. S., SHELL, R., SFERRA, T. J., CHIRMULE, N., MAGOSIN, S. A., PROPERT, K. J., BROWN-PARR, E. C., HUGHES, J. V., TAZELAAR, J., BAKER, C., GOLDMAN, M. J. & WILSON, J. M. 1999. A phase I study of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator gene to a lung segment of individuals with cystic fibrosis. *Human gene therapy,* 10**,** 2973-85.

ZUGHAIER, S. M., RYLEY, H. C. & JACKSON, S. K. 1999. Lipopolysaccharide (LPS) from Burkholderia cepacia is more active than LPS from Pseudomonas aeruginosa and Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes. *Infection and immunity,* 67**,** 1505-7.

10.1 World Wide Web sources

www.genet.sickkids.on.ca/cftr, entered October 2011

www.genet.sickkids.on.ca/treatments, entered September 2011

www.cftrust.org.uk, entered October 2011

www.tracheostomy.com/resources/anatomy/index.htm (figure 1.1), entered October 2011

www.biomin.net/es/soluciones/endotoxin-risk-management/; entered October 2011

www.pharmaceutical-int.com/article/trans-epithelial-electric-resistance-teer-measurements; entered October 2011

11 Conferences attended

March 2009	APS Inhalation, Academy of Pharmaceutical Sciences Nottingham, UK
April 2010	European Cystic Fibrosis Society, Basic Science Conference, Carcavelos, Portugal
October 2010	24th Annual North American Cystic Fibrosis Conference, Baltimore, USA
June 2011-10-25	34th European Cystic Fibrosis Conference Hamburg, Germany

12 List of Publications

12.1 Full papers

Apoptotic cell-derived ICAM-3 promotes both macrophage chemoattraction to and tethering of apoptotic cells.

Elizabeth E. Torr[1], David H. Gardner[1], Leanne Thomas[1], D. Margaret Goodall[3], **Anne Bielemeier[1]**, Rachel Willetts[1], Helen R. Griffiths[1,2], Lindsay J. Marshall[1,2] and Andrew Devitt*[1,2] (publishing in proceedings)

12.2 Abstracts

Bielemeier, A., Marshall, L. J.

Development of a multi-cellular co-culture model of normal and cystic fibrosis human airways

Published in Pediatric Pulmonology, Supplement 33, 2010